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Examination of Microsporidia Spore Adherence and Host Cell Infection *In Vitro*

A dissertation
presented to
the faculty of the Department of Microbiology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences

by
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May 2007

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Adherence, Infectivity, Microsporidia Attachment Protein, MsAP

ABSTRACT

Examination of Microsporidia Spore Adherence and Host Cell Infection *In Vitro*

by

Timothy R. Southern

Microsporidia are obligate intracellular pathogens that cause severe disease in immunocompromised humans. While albendazole is the treatment of choice, no therapy exists that effectively treats all forms or causes of human microsporidiosis. Recent studies show that the microsporidian *Encephalitozoon intestinalis* binds glycosaminoglycans (GAGs) associated with the host cell surface, and that the divalent cations manganese (Mn^{2+}) and magnesium (Mg^{2+}) augment spore adherence to host cells by activating a constituent on the spore surface. These studies also illustrate a direct relationship between spore adherence and host cell infection; inhibition of spore adherence leads to reduced host cell infection while augmentation of spore adherence increases host cell infection. In light of recent studies, microsporidia spore adherence has become a promising target for the development of novel therapeutics to treat or even prevent human microsporidiosis. The goal of this study was to further characterize the molecular mechanisms governing spore adherence by identifying specific constituents on microsporidia spores that participate in spore adherence with host cells. A 40 kDa *Encephalitozoon cuniculi* host cell binding protein was discovered and identified as ECU01_0820, hereafter known as *Encephalitozoon cuniculi* microsporidia spore adherence protein (EcMsAP). The

gene encoding EcMsAP has multiple heparin-binding motifs and an integrin-binding domain, which are characteristic of proteins that interact with constituents on the cell surface. Immuno-transmission electron microscopy reveals that native EcMsAP is located on the plasma membrane, endospore, exospore, and the anchoring disk of microsporidia spores. Recombinant EcMsAP and antibodies to recombinant EcMsAP both inhibit spore adherence and host cell infection. However, the deletion of heparin-binding motif #1 from the EcMsAP gene results in the loss of ability to inhibit spore adherence and infection. Host cell-binding assays reveal that recombinant EcMsAP binds Vero and CHO cell lines but exhibits attenuated binding to glycan-deficient CHO cell lines. Finally, biomolecular interactions analysis provides direct evidence that EcMsAP is a glycan binding protein. This study not only identifies a potential microsporidial vaccine candidate, it further supports the assertion that microsporidia spore adherence is a critical step in the host cell infection process.

DEDICATION

To family and friends lost along the way.

ACKNOWLEDGMENTS

This dissertation and the years of work it represents would not have been possible without the love and support of my family. Their constant dedication to my academic endeavors gave me the strength to succeed.

To my wife Carrie Elizabeth Jolly: Thank you for tolerating my antics and tantrums in and out of the laboratory. I look forward to a lifetime of 'love and happiness' together.

To my daughter Brooklyn Elise Boster: Thank you for spending so many weekends in the laboratory and for reminding me that sometimes I need to loosen up and be a kid again.

To my committee: The past five years would not have been possible without your support. Your dedication to my education has equipped me to take the next step toward a career as an independent scientist.

This research was supported by a grant to James Russell Hayman from the National Institutes of Health and by a grant to Timothy R. Southern from the East Tennessee State University College of Graduate Studies.

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CHAPTER 1

INTRODUCTION

Microsporidia are obligate intracellular opportunistic pathogens that infect a variety of hosts including humans. Thirteen species of microsporidia belonging to eight genera are known to infect humans. Although the first reported case of human microsporidiosis was in 1959 (Matsubayashi et al. 1959), it was not until the onset of the AIDS epidemic that microsporidia were recognized as important human pathogens (Desportes et al. 1985). *Enterocytozoon bieneusi* and *Encephalitozoon* spp. are the most common causes of human microsporidiosis (Molina et al. 1993; Weber et al. 1994).

First described in 1985, *Enterocytozoon bieneusi* is the most frequent cause of severe diarrhea in HIV patients (Canning and Hollister 1992; Didier et al. 1998). *E. bieneusi* also causes disseminated infections including sinusitis, rhinitis, bronchitis, and pneumonia in the immunosuppressed (Weber et al. 1992; Weber et al. 1994). Only a few cases of microsporidiosis in patients without HIV infection are recorded for *E. bieneusi*. These include cases of self-limiting diarrhea in a liver transplant recipient (Sandfort et al. 1994) and an otherwise healthy individual who likely contracted microsporidiosis from travels abroad (Weber et al. 1994).

Several *Encephalitozoon* species also cause human microsporidiosis. Mammals, including rodents, carnivores, and primates are infected by

Encephalitozoon cuniculi suggesting zoonotic transmission to humans (Slifko et al. 2000; Wasson and Peper 2000; Weiss 2001). *E. cuniculi* causes severe diarrhea, fulminant hepatitis, peritonitis, and a myriad of other disseminated infections and is reported to have a predilection for cells in the brain and kidneys (Canning et al. 1986).

Encephalitozoon hellem and *E. intestinalis* are also common parasites of HIV infected humans. *E. hellem* was first described in 1991 from three AIDS patients with keratoconjunctivitis (Didier et al. 1991). *E. hellem* also causes disseminated infections including nephritis, bronchiolitis, and pneumonia (Schwartz et al. 1992; Schwartz et al. 1993; Schwartz et al. 1993). *E. intestinalis* was first described in 1993 as *Septata intestinalis* (Cali et al. 1993) and causes severe diarrhea, nephritis, and cholecystitis (Weber et al. 1994).

Treatment of Human Microsporidiosis

Most cases of human microsporidiosis are treated with albendazole, a broad-spectrum antiprotozoal benzimidazole that disrupts microtubule polymerization in the developing parasite (Ridoux and Drancourt 1998). Fumagillin, although highly toxic, is also used to treat ocular microsporidial infections (Didier 1997; Ridoux and Drancourt 1998). A less toxic fumagillin derivative, TNP-470, has shown promise as a systemic treatment for microsporidiosis (Didier 1997). While albendazole and fumagillin are the most common treatments for microsporidiosis, they are at best variably effective (Didier et al. 2006). Novel compounds including Ovalicin and Ovalicin derivatives

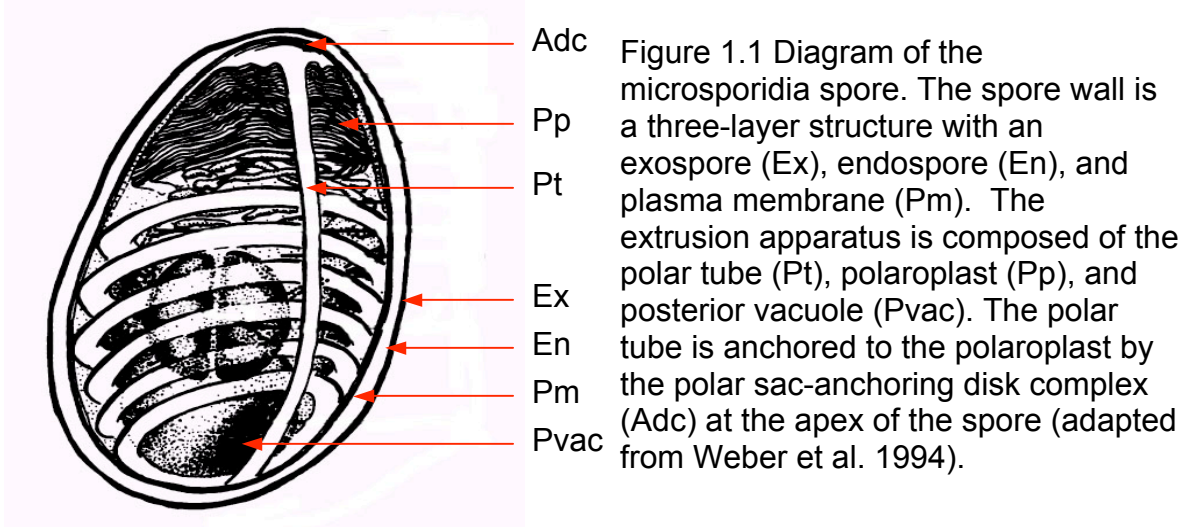
have also shown promise as potential therapies for microsporidiosis (Didier et al. 2006). Unfortunately, no single compound can be used to treat all causes or forms of human microsporidiosis.

Spore Structure

The environmentally resistant spore is the only infectious form of microsporidia. The spore is encased in a three-layer wall composed of an exospore, endospore, and plasma membrane (Figure 1.1) (Wittner 1999). Little is known about the composition of the microsporidia spore wall except that is partially composed of chitin or a chitin-like material, contains a protein component, and is structurally rigid. Unlike the spore wall, the internal ultrastructure of the microsporidia spore is well described. All microsporidia possess a hollow polar tube that is used to infect host cells. The polar tube is associated with the polaroplast, a voluminous structure that comprises one-third to one-half of the total spore volume. Microsporidia spores also possess a posterior vacuole, a membranous vesicle located at the posterior end of the mature spore. Together, the polar tube, polaroplast, and posterior vacuole form the extrusion apparatus responsible for polar tube discharge and delivery of infectious sporoplasm to the host cell (Wittner 1999).

Also of note is the polar sac-anchoring disk complex that secures the polar tube to the polaroplast at the anterior-most portion of the spore where the spore wall is thin. Microsporidia spores also contain a full complement of eukaryotic membrane-bound organelles including one or more nuclei, ribosomes, Golgi, and

an endoplasmic reticulum, among others. Of particular interest, all microsporidia lack mitochondria yet possess mitochondrial heat shock proteins suggesting a secondary loss of the mitochondria during microsporidial evolution (Hirt et al. 1997). This condition is characteristic of ancient eukaryotic organisms and was once used to categorize microsporidia as protozoa. However, recent phylogenetic analysis indicates that microsporidia are highly derived fungal pathogens (Gill and Fast 2006). Also of interest, microsporidial ribosomal RNA molecules have sedimentation coefficients of prokaryotic ribosomal RNA (Wittner 1999). Loss of mitochondria and development of ribosomal RNA with prokaryotic affinities are likely results of the selective pressures of an obligate intracellular lifestyle.



Microsporidia Spore Activation and Host Cell Infection

Medically important microsporidia of the genus *Encephalitozoon* are typically introduced to the host digestive system following ingestion of contaminated water or food (Slifko et al. 2000). Based on an unknown stimulus,

an *Encephalitozoon* spore becomes activated leading to polar tube discharge and host cell infection. Initial activation of the spore leads to a calcium-induced increase in the internal pressure of the polaroplast (Weidner and Byrd 1982). Calcium efflux from the swollen polaroplast then causes the polar tube to be forcefully discharged from the apical terminus of the spore. As it leaves the spore, the polar tube everts, or is forced inside-out revealing an outer polar tube surface that is decorated with polar tube proteins. The everted polar tube ultimately penetrates the host cell plasma membrane. The release of pressure from the polaroplast also forces infectious sporoplasm through the everted polar tube and into the host cell where a new generation of spores develops (Weidner and Byrd 1982).

Intracellular Development of *Encephalitozoon* spp.

Once delivered to the host cell, infectious sporoplasm gives rise to the meront, the first of four microsporidial forms (Figure 1.2). The meront is an irregularly shaped microsporidial form that possesses at least one distinct nucleus and evidence of a developing endoplasmic reticulum. Meronts develop at the periphery of the parasitophorous vacuole (PV), and appear electron-lucent when viewed by transmission electron microscopy (TEM). Meronts mature into sporonts that are characterized by a thickening outer wall but are otherwise ultrastructurally similar to meronts. Sporonts also reside at the margins of the PV and are electron-lucent compared to mature forms. Sporonts eventually mature into sporoblasts, which exhibit a continuous plasma membrane, a developed

spore wall, a full complement of organelles, and early evidence of an extrusion apparatus (polar tube, polaroplast, and posterior vacuole). Sporoblasts develop within the lumen of the PV and appear electron-dense compared to meronts and sporonts when viewed by TEM. Finally, fully mature spores are formed following organelle polarization. Mature spores are also electron-dense and reside in the lumen of the vacuole. Microsporidia spore egress, a poorly understood process, occurs following spore maturation and results in host cell death (del Aguila et al. 1998; del Aguila et al. 2001).

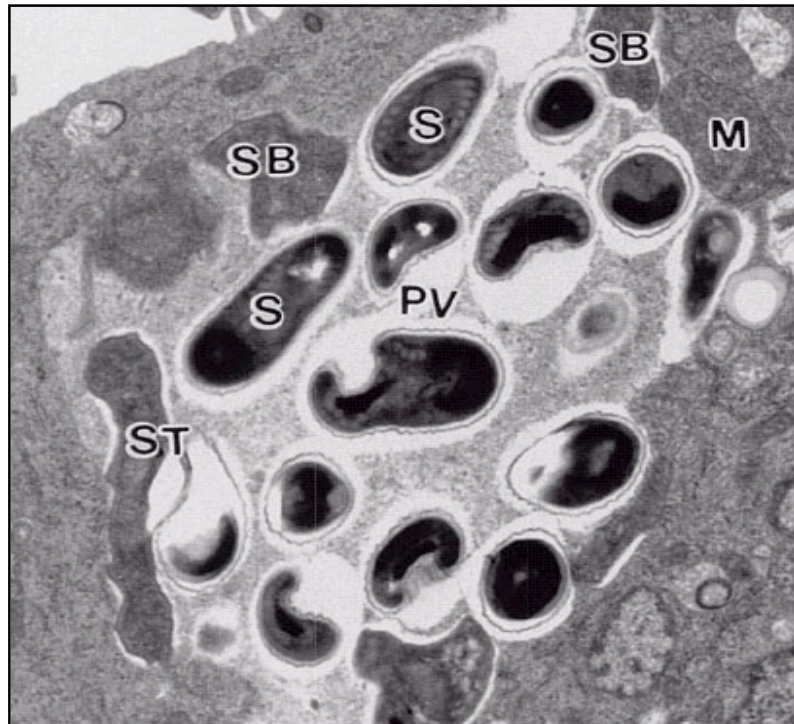


Figure 1.2 Electron micrographs of developing *Encephalitozoon* meronts (M), sporonts (ST), sporoblasts (SB), and spores (S) contained within a parasitophorous vacuole (PV) (image adapted from del Aguila et al. 1998).

Specific Aims

Little is known about the molecular events that govern microsporidia spore activation and the initiation of host cell infection. One hypothesis suggests that the physical interaction between the microsporidia spore and host cell is the stimulus required to activate the spore. Otherwise known as spore adherence, this phenomenon occurs *in vitro* during spore propagation and can be viewed by light microscopy (personal observations). Of particular interest to our laboratory are microsporidial pathogens of the genus *Encephalitozoon*. *Encephalitozoon intestinalis*, a pathogen important to human health, is an example of a microsporidian that binds avidly to the surface of host cells during *in vitro* culture.

The overall goal of this research is to better characterize the molecular mechanisms that govern microsporidia spore adherence to host cells *in vitro*. The specific aims of this research are to (1) identify potential host cell receptors that bind spores *in vitro*, (2) examine the role of divalent cations in spore adherence, and (3) identify potential microsporidia spore ligands that participate in spore adherence to *in vitro* grown host cells.

CHAPTER 2

THE ROLE OF GLYCOSAMINOGLYCANS IN ADHERENCE OF THE MICROSPORIDIAN, *ENCEPHALITOZOON INTESTINALIS*, TO HOST CELLS *IN VITRO*

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Running Title: Microsporidia Spore Adherence to Host Cells

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Hayman, J. R., Southern, T. R. and Nash, T. E. (2005). Role of sulfated glycans
in adherence of the microsporidian *Encephalitozoon intestinalis* to host
cells *in vitro*. *Infect Immun* 73(2): 841-8.

Abstract

Microsporidia are obligate intracellular opportunistic protists that infect a wide variety of animals via environmentally resistant spores. One of the more common causes of microsporidiosis in humans is *Encephalitozoon intestinalis*. Infection requires that spores be in close proximity to host cells so that the hollow polar tube can pierce the cell membrane and inject the spore contents into the cell cytoplasm. Like other eukaryotic microbes, microsporidia may use a specific mechanism(s) for adherence in order to achieve target cell proximity and increase the likelihood of successful infection. Our data show that *E. intestinalis* exploits glycosaminoglycans (GAGs) in selection of and attachment to host cells. When exogenous sulfated GAGs are used as inhibitors in a spore adherence assay, *E. intestinalis* spore adherence is reduced as much as 88%. However, there is no inhibition when non-sulfated GAGs are used, suggesting that *E. intestinalis* spores use sulfated host cell GAGs in adherence. These studies were confirmed by exposure of host cells to xylopyranoside or sodium chlorate treatments, treatments that limit host cell surface GAGs and decreases surface sulfation, respectively. Spore adherence studies using the CHO mutant cell lines either deficient in surface GAGs, or deficient in surface heparan sulfate indicate a preference of *E. intestinalis* spores for heparan sulfate. Furthermore, when spore adherence is inhibited, host cell infection is reduced, indicating a direct association between spore adherence and infectivity. These data show that *E.*

intestinalis specifically adheres to target cells by way of sulfated host GAGs and this mechanism serves to enhance infectivity.

Key words: Adherence, Infectivity, Glycosaminoglycan, *Encephalitozoon intestinalis*

Introduction

Microsporidia are obligate intracellular opportunistic protists that infect vertebrates and invertebrates alike. Although microsporidia were identified as agents of disease in animals more than 150 years ago, it was during the AIDS epidemic that microsporidia were implicated as a cause of the severe diarrhea and systemic infections seen in some HIV infected individuals. However, microsporidiosis is not limited to the immunosuppressed as there are numerous reports of immunocompetent persons becoming infected (Svenungsson et al. 1998; Lopez-Velez et al. 1999; Visvesvara et al. 1999). Most human microsporidiosis is due to infection with *Enterocytozoon bieneusi*, while the second most common is *Encephalitozoon intestinalis* (Kotler and Orenstein 1999). An efficient long-term *in vitro* culturing method for *E. bieneusi* has not been established; therefore, *E. intestinalis* is commonly used to study microsporidiosis (Visvesvara et al. 1999).

Microsporidia are transmitted via an environmentally stable spore and infect host cells by a unique mechanism. It is thought that when an ingested spore comes in close association with a host cell in the gastrointestinal tract, it encounters the optimal conditions for spore activation triggering a cascade of events leading to the extrusion of a hollow polar filament that pierces the host cell plasma membrane (Cali and Takvorian 1999). The infectious sporoplasm is then injected into the host cell cytoplasm where the parasite subsequently propagates. When developing spores mature, the host cell ruptures releasing them into the lumen of the gut to be excreted back into the environment, infect nearby cells, or,

as in the case of *E. intestinalis*, disseminate to other tissues and organs throughout the body (Cali et al. 1993). Microsporidia spores may also be internalized by both professional and non-professional phagocytes via an actin-based mechanism (Weidner and Sibley 1985; Couzinet et al. 2000). However, regardless of whether spores are internalized or extra-cellular, host cells are not known to become infected without spore polar filament extrusion.

Because of the obligate intracellular nature of microsporidia, most are routinely cultured and propagated with host cells *in vitro* (Visvesvara et al. 1999). During *in vitro* cultivation, microsporidia spores are generally recognized to adhere to host cell surfaces and adherent spores cannot be removed by routine washing. Although this adherence seems to occur spontaneously, the mechanism of adherence has not been described. Therefore, this study examines spore adherence to host cells to determine if a specific mechanism is involved.

In studies of other eukaryotic microbes, glycosaminoglycans (GAGs), proteoglycans that are found on almost all cell types, have been shown to play important roles in selection of and attachment to host cells. *Plasmodium falciparum* sporozoites, for example, bind heparin and heparan sulfate, allowing them to target hepatocytes and the placenta (Frevert et al. 1993; Wadstrom and Ljungh 1999). And the broad host and tissue recognition of *Toxoplasma gondii* is attributed to the ability of the parasite to bind a variety of host GAGs (Carruthers et al. 2000). Therefore, it was of interest to determine the role of host cell GAGs in adherence of *E. intestinalis* spores.

Materials and Methods

Microsporidia and Host Cell Cultivation.

Adherent host cell lines used for cultivation of microsporidia spores and as substrate for spore adherence assays included African green monkey kidney cells (Vero; ATCC number CCL-81), rabbit kidney cells (RK-13; ATCC number CCL-37), human epithelial colorectal cells (Caco-2; ATCC number HTB-37), chinese hamster ovary cells (CHO, ATCC number CCL-61), mutant CHO pgsA-745 cells (ATCC number CRL-2242), and mutant CHO pgsD-677 cells (ATCC number CRL-2244). All cell lines were grown as previously described (Hayman and Nash 1999) in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml), and 10 % fetal bovine serum (FBS) (BioWhittaker) in 5% CO₂ at 37°C. For maintenance of Vero and RK cells, medium with 10% FBS was replaced with 2% FBS.

For microsporidia spore propagation, subconfluent Vero monolayers in T-75cm² flasks were infected with *E. intestinalis* spores as previously described (Hayman et al. 2001). Briefly, spores were incubated with the adherent host cells for 12 to 15 days with medium replacement every two or three days. Spores were then harvested from the flasks daily until most host cells were dead. The spores were purified from host cell debris by washing once with 0.25% sodium dodecyl sulfate (SDS), followed by several washes with sterile water. Spore stocks were counted and stored at 4°C.

Spore Adherence Assays

Host cells were seeded on glass coverslips (18 mm size) at 5×10^5 in 12 well plates with normal growth medium and allowed to grow for a minimum of 16 hours. Then, 1 or 10 million *E. intestinalis* spores were added to each well with 1 ml of fresh medium supplemented with 1 mM MnCl_2 for 4 hours either on ice or at 37°C . In the spore adherence time course experiment, the incubation period ranged from 2 to 48 hours. The spore inocula represent an approximate multiplicity of infection of 2 and 20, respectively. The coverslips were then harvested and washed with PBS to remove any non-adherent spores. Dried coverslips were fixed with acetone/methanol and an immunofluorescent assay (IFA) was performed to quantitate the number of bound spores. The monoclonal antibody 7G7 (Lujan et al. 1998), which recognizes SWP2 of *E. intestinalis* (Hayman et al. 2001), was used as the primary antibody, and a fluoresceine isothiocyanate conjugated anti-mouse was used as the secondary antibody (Rockland Immunochemicals). Fluorescent microscopy (Zeiss; Axiovert S100) was employed to count the number of bound spores per field of magnification at 630X and the results were expressed as the mean \pm standard deviation or in some experiments as the percentage of adherent spores relative to control samples is shown.

The glycosaminoglycans (GAGs) that were tested as potential inhibitors of spore adherence included heparin (Sigma), chondroitin sulfate A (CSA; Sigma), chondroitin sulfate B (CSB; Sigma), type II mucin (Sigma), dextran sulfate (Sigma), and dextran (Sigma). Each GAG was dissolved in maintenance

medium supplemented with 1 mM MnCl_2 at the maximal indicated concentration and was serially diluted. The diluted GAGs were added with the spores to Vero host cell grown on glass coverslips as described for the adherence assay. Spore adherence was quantitated by IFA and the percentage of adherent spores was calculated. Effective concentration values (EC_{50}), defined as the concentration of inhibitor that produces a 50% fall of spore adherence from 100% to an asymptotic value, were calculated by generating standard curves from the spore inhibition data with the assistance of the SAS/PC statistical software (SAS Institute, Inc).

To confirm the role of host cell surface GAGs in spore adherence, Vero host cells were grown on glass coverslip in the presence of 1 or 10 mM p-nitrophenyl- β -D-xylopyranoside for 24 hours prior to the addition of 10 million spores per well. Xylopyranoside acts as a soluble acceptor for GAG polymerization and will compete with the endogenous proteoglycan core assembly, resulting in an absent or diminished amount of surface proteoglycans (Mark et al. 1990). In control samples, p-nitrophenyl- α -D-galactopyranoside, which does not affect GAG assembly, was substituted.

The adherence assay was also performed using the CHO mutant cell lines pgsA-745 (ATCC# CRL-2242) and pgsD-677 (ATCC# CRL-2244) and the non-mutated CHO-K1 parent cell line (ATCC# CCL-61). These mutant cell lines are deficient or have reduced surface GAGs (Esko et al. 1985; Esko et al. 1988). After seeding the cells on glass coverslips, 10 million spores were added to the

mutant and parent cell lines in 1 ml of medium for 4 hours on ice. The coverslips were removed, washed and fixed and an IFA was performed as described.

The role of host cell surface GAG sulfation was confirmed by exposing Vero host cells to 10, 20, 40, or 60 mM NaClO₃, or NaCl₂ as a control, for 24 or 48 hours prior to the addition of 10 million *E. intestinalis* spores. The standard adherence assay was performed and the percentage of adherent spores were calculated relative to untreated control samples.

Rate of Infection in the Presence of Inhibitor

Using Vero host cells grown on glass coverslips, the spore adherence assay was performed in the presence of 10 µg/ml CSA for 4 hours. The inoculum ranged from 1 to 100 million spores per well of a 12 well culture plate. Control coverslips were removed after 4 hours and processed by IFA to measure the percentage of inhibition of spore adherence in the presence or absence of CSA. The remaining coverslips were washed with PBS to remove unbound spores and placed back in culture for about 30 hours to allow infection and intracellular spore propagation to occur. The developing intracellular spore clusters were visualized by propidium iodine staining, and the percentage of infected cells was determined by dividing the number of infected cells by the total number of host cells per field of 630X magnification.

Transmission Electron Microscopy (TEM)

Caco-2 intestinal epithelial host cells were seeded at 1×10^5 on 8 μm porous transwell inserts (BD Biosciences) in growth medium and were maintained for at least 7 days prior to experimentation. Fifty million *E. intestinalis* spores were added to the upper chamber and were allowed to adhere to the host cell surfaces for 8 hours. The unbound spores were removed by washing and the host cells were prepared for transmission electron microscopy as previously described (Guseva et al. 2003). Briefly, the host cell / spore sample was fixed in 2% glutaraldehyde, 0.5% paraformaldehyde in 0.1 M cacodylate buffer for 2 hours at 37°C. The host cells were then scraped from the inserts, pelleted, and enrobed in 3% SeaKem agarose. After washing the host cell pellet in 0.2 M cacodylate buffer containing 0.3 M sucrose, it was post-fixed for 1 hour at 25°C in 1% osmium tetroxide in 0.2 M cacodylate buffer. The sample was sequentially dehydrated in ethanol, infiltrated with Epon-Araldite 812 resin, and embedded in fresh Epon. Ultra-thin sections were cut on a Reichert Ultracut (Leica) microtome and subsequently mounted and viewed with a Tecnai-10 electron microscope.

Results

Microsporidia Spore Adherence to Host Cells

Encephalitozoon intestinalis can be cultured easily *in vitro* using a variety of adherent cell lines (Visvesvara et al. 1999). Once in culture, the spores adhere to the surface of host cells as observed by light microscopy. The adherent spores cannot be removed by routine washing. To examine spore

adherence more closely, TEM was performed using *E. intestinalis* spores and the human intestinal epithelial cell line Caco-2 (Figure 2.1A). Many adherent spores were observed in direct physical contact with both the host cell surface and microvilli. Although it is sometimes difficult to determine the orientation of adherent spores because of the ultra-thin sections used in transmission electron microscopy, some spores were clearly attached longitudinally to the cell surface. Other spores were oriented such that the apical end of the spore, where the anchoring disk is located, was in close approximation to the host cell (Figure 2.1B). Orientation in this manner would allow for the extrusion of the polar filament directly into the host cell. However, many retained the polar filament in the characteristic coiled nature within the spore, despite attachment in this orientation. This may indicate that spore adherence to host cell surfaces is an event that is a precursor to spore activation perhaps leading to polar filament extrusion. Alternatively, it is possible that the attached spores are incapable of polar filament discharge.



Figure 2.1 TEM of *E. intestinalis* spores attached to Caco-2 cell surfaces. *E. intestinalis* spores were allowed to adhere to the apical surface of confluent Caco-2 cells grown on porous transwell inserts. After a 4-hour incubation of host cells with 1×10^7 spores, the unbound spores were removed by washing, and the transwell inserts were fixed and processed for TEM as described. The attached spores appear to be in direct contact with the cell surface or microvilli and have either intact, un-extruded polar filaments (A) or extruded polar tubes (B).

Rate of *E. intestinalis* Spore Adherence to Host Cells

An adherence assay was developed to quantitate spore adherence to host cells. The rate of *E. intestinalis* spore adherence to the surface of the host cell was measured, and the peak in spore adherence occurred about 8 hours post inoculation followed by a slight decrease and plateau in adherence over the next 40 hours (Figure 2.2). At the peak of adherence in either Vero or RK cells, approximately 800 spores were observed per field of magnification. This equates to approximately 10 spores per cell. Whether this represents a saturation of spore adherence to host cell surfaces or the limit of adherence capable spores in the inoculum is unclear; however, these calculations indicate that approximately

one-tenth of the inoculum was attached in these experiments. The decrease in spore adherence beginning after 8 hours incubation may indicate phagocytosis and subsequently degradation by the host cell. Alternatively, it may indicate attachment and release of spores from the host cell surface.

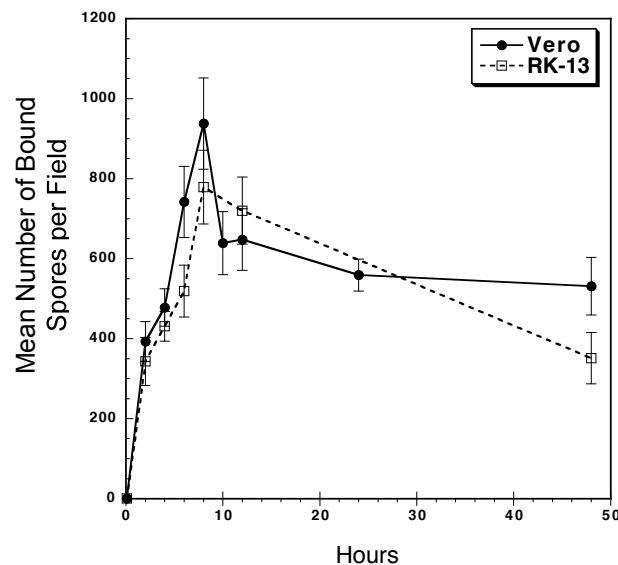


Figure 2.2 The rate of *E. intestinalis* spore adherence to Vero and RK host cells. An adherence assay was performed with 1×10^7 spores and either Vero or RK cells grown on glass cover slips in 12 well plates. The cover slips were removed at the indicated times and washed. The bound spores were quantitated by immunofluorescences as described. Twenty random magnification fields for each data point were counted and the mean number of attached spores was determined for each indicated time point. These data represent one experiment of three that were performed with similar results.

Inhibition of Spore Adherence by Exogenous Sulfated Glycosaminoglycans

GAGs are found on the surface of almost all vertebrate cell lineages (Bernfield et al. 1992; Yanagishita and Hascall 1992). The involvement of GAGs in adherence of other pathogens to host cells, leading to invasion and infection, has been well documented (Bernfield et al. 1999). To determine if GAGs play a

role in microsporidial spore adherence to host cells, various exogenous GAGs were used as inhibitors of adherence. In this experiment, heparin, CSA, CSB, mucin, and dextran sulfate inhibited *E. intestinalis* adherence to host cells, implicating the involvement of host cell surface GAGs in *E. intestinalis* spore adherence to host cell surfaces (Figure 2.3). The inhibition was dose dependent, resulting in about 73-88% reduction in spore adherence when higher concentrations of the exogenous proteoglycans were used as compared to control samples without exogenous proteoglycans. Furthermore, dextran sulfate inhibited spore adherence in a dose dependent manner, whereas, non-sulfated dextran did not. These results suggest that spore adherence to host cells involves a specific GAG dependent mechanism which is limited to sulfated GAGs.

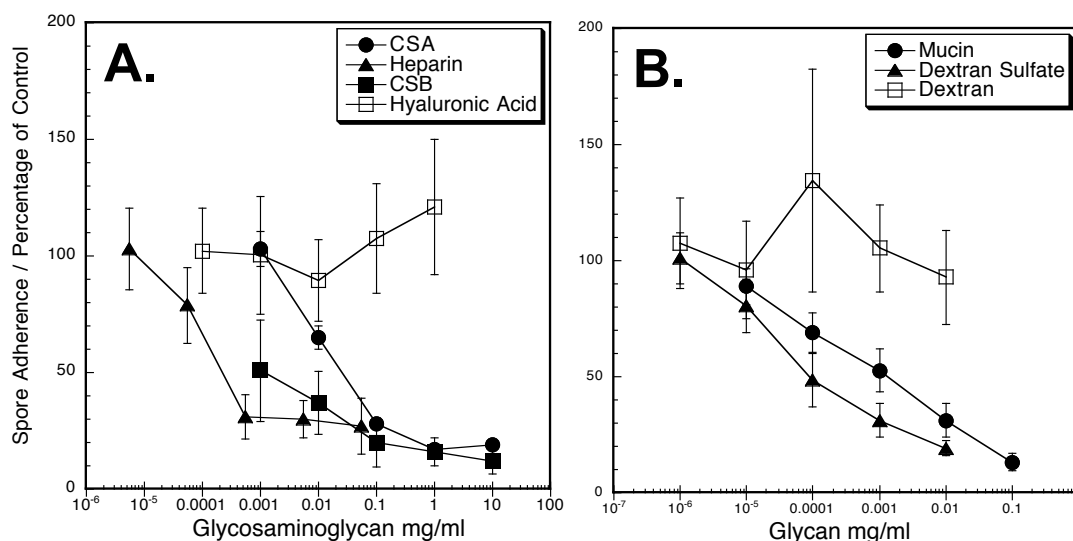


Figure 2.3 *E. intestinalis* spore adherence is inhibited by exogenous sulfated glycans. An adherence assay was performed as described in the presence or absence of various exogenous glycans in 10 fold serial dilutions. The glycans tested as potential inhibitors include the GAGs heparin, chondroitin sulfate A, chondroitin sulfate B, and hyaluronic acid (A). The non-GAG glycans tested include porcine stomach mucin, dextran sulfate, and dextran (B). After four hours of incubation of 1×10^7 spores with or without the glycan inhibitor, the host cells grown on coverslips were removed and washed of unbound spores. The mean number of attached spores was determined for each concentration of potential inhibitor. The percentage of spores inhibited from adherence is shown relative to control samples without exogenous glycans. The data presented here represent one experiment of three that were performed with similar results.

To evaluate and compare effective concentrations of GAG inhibitors, standard curves were generated for each GAG tested and the EC_{50} values were determined (Table 2.1). EC_{50} values are defined as the effective concentration that produces a 50% fall of spore adherence from 100% to an asymptotic value. These data show that heparin is the most efficient and dextran sulfate is the least efficient of the inhibitors tested. However, when the standard curves were generated, it was evident that dose dependent inhibition leveled off revealing the maximal inhibition regardless of inhibitor concentration (i.e. peak reduction).

These data indicate that while heparin is the most efficient at inhibiting spore adherence, the maximal level of inhibition achieved is approximately 28% of the control, whereas the estimated maximal level of inhibition for mucin is approximately 1.5% of control.

Table 2.1 EC₅₀ Values for Exogenous GAG Inhibitors of Spore Adherence

GAG	EC ₅₀ ^a	95% Confidence Interval		Estimated Peak Reduction ^b
	µg/ml	Lower	Upper	µg/ml (+/- SE ^c)
Heparin	0.097	0.059	0.157	28.4 (1.38)
CSA ^d	14.08	6.44	30.78	18.0 (2.72)
CSB ^e	0.641	0.193	2.13	11.2 (2.99)
Mucin	0.119	0.003	4.87	1.5 (13.33)
Dextran Sulfate	53.67	19.45	414.11	15.3 (4.46)
Dextran	No Response Curve			

^a EC₅₀ is defined as effective concentration that produces a 50% fall of spore adherence from 100% to the asymptotic value (i.e. Peak Reduction).

^b Peak Reduction is the lowest concentration of inhibitor calculated to achieve the maximal inhibition.

^c Standard Error.

^d Chondroitin Sulfate A

^e Chondroitin Sulfate B

Confirmation of Sulfated Glycosaminoglycan Mediated Spore Adherence

To confirm that *E. intestinalis* spore adherence involves surface GAGs, host cells were grown in the presence of p-nitrophenyl-β-D-xylopyranoside for 24 hours prior to performing the adherence assay. p-nitrophenyl-β-D-xylopyranoside acts as a soluble receptor for GAG polymerization and will compete with endogenous proteoglycan core assemblies, resulting in an absent or diminished amount of surface proteoglycans (Mark et al. 1990). Following host cell treatment with xylopyranoside, *E. intestinalis* spore adherence was

reduced by 90% compared to untreated host cells (Figure 2.4). As a control, host cells were also treated with p-nitrophenyl- α -D-galactopyranoside, which does not affect GAG assembly (Mark et al. 1990); therefore, the host cells have a full complement of surface GAGs. The data show no significant difference compared to untreated host cells.

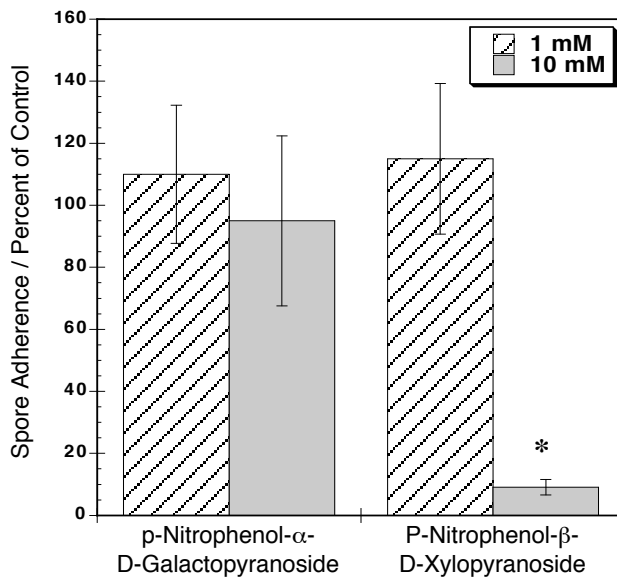


Figure 2.4 *E. intestinalis* spore adherence to host cells with reduced surface proteoglycans following xylopyranoside treatment. The standard adherence assay was performed using Vero host cells that were treated with normal medium supplemented with either 1 or 10 mM p-nitrophenyl- β -D-xylopyranoside or control p-nitrophenyl- α -D-galactopyranoside for 24 hours prior to the assay. This experiment was repeated three times with similar results. Asterisk indicates significance at $p < 0.0001$ using the Student's t-test.

The spore adherence assay was also performed with non-mutant parent and mutant CHO cell lines that lack specific GAG assemblies (Esko et al. 1985; Esko et al. 1988). Spore adherence to the pgsA-745 mutant cells that are completely surface GAG deficient was reduced 94% in comparison to control (Figure 2.5). Interestingly, using the mutant cell line pgsD-677 that lacks surface

heparan sulfate but has three-fold higher levels of chondroitin sulfate also results in a 91% reduction in adherence. These data indicate that *E. intestinalis* preferentially binds host cell surface heparan sulfate and confirm that *E. intestinalis* spore adherence is dependant on host cell surface GAGs.

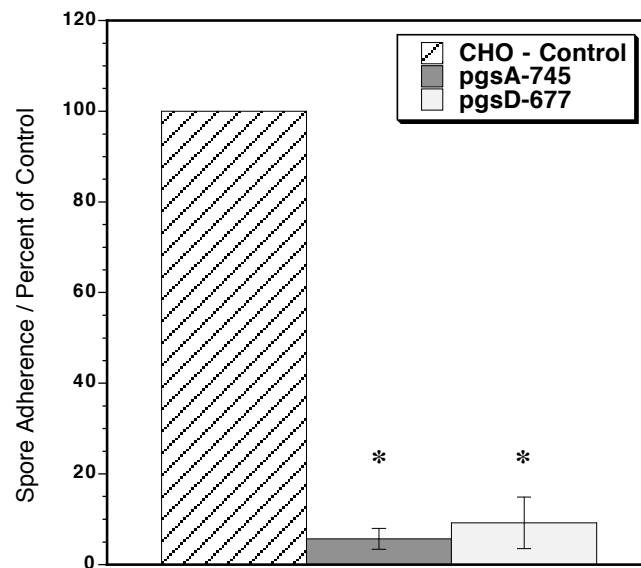


Figure 2.5 *E. intestinalis* spore adherence to host cells with reduced surface proteoglycans. The standard adherence assay was performed using CHO pgsA-745 and pgsD-677 mutant cell lines in addition to the control non-mutated CHO parent cell line. This experiment was repeated four times with similar results. The data are shown as the percentage of spore adherence relative to control samples of non-mutated parent CHO cells. Twenty random fields were counted for each data point. The data presented are representative of three independent experiments. Asterisk indicates significance at $p < 0.0001$ using the Student's t-test.

In additional experiments, host cells were treated with sodium chlorate that significantly reduces sulfation of surface chondroitin and heparan without significantly altering host cell growth (Humphries and Silbert 1988). When host cells are treated with 10 to 60 mM sodium chlorate for 24 or 48 hours prior to the adherence assay, *E. intestinalis* spore adherence is reduced approximately 40%

to 80%, respectively (Figure 2.6). Host cells treated with equivalent concentrations of sodium chloride showed no inhibition of spore adherence as compared to untreated host cells. These data confirm that microsporidia spore adherence to host cells is dependent on sulfated surface GAGs.

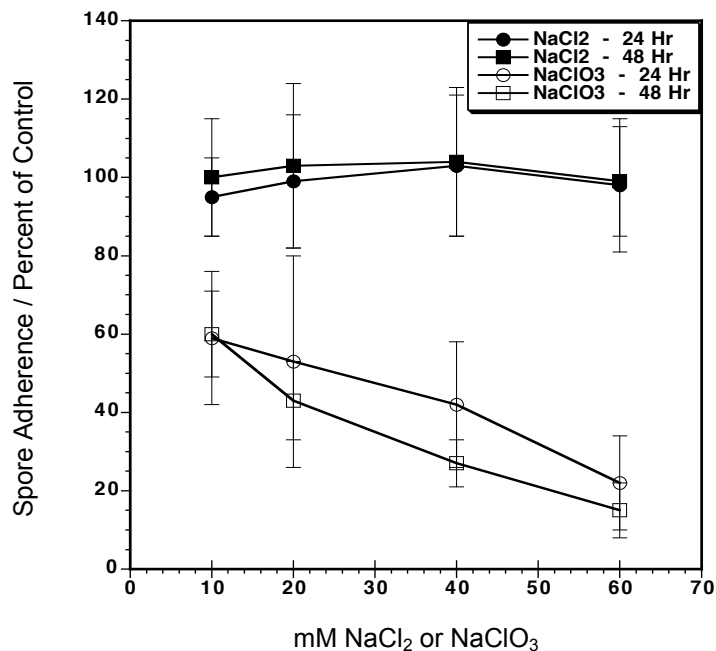


Figure 2.6 *E. intestinalis* spore adherence to host cells with reduced sulfated surface proteoglycans following sodium chlorate treatment. Vero host cells grown on glass cover slips in 12 well plates were treated with 10, 20, 40, or 60 mM sodium chlorate or control sodium chloride for 24 or 48 hours prior to the standard spore adherence assay. The percentage of attached spores relative to spore adherence on non-treated host cells is shown. All sodium chlorate treated samples are significantly different than either sodium chloride or non-treated controls ($p < 0.0001$). The data presented are representative of three experiments that were performed with similar results.

Inhibition of *E. intestinalis* Spore Adherence Results In Decreased Infection

To determine the role of spore adherence in infection, the number of infected host cells was quantified after *E. intestinalis* spores were allowed to adhere to host cell surfaces in the presence or absence of an inhibitor (Figure

2.7A) Following adherence in the presence or absence of the inhibitor, the unbound spores were removed by washing. The coverslips were placed back in culture without inhibitor, and the infection was allowed to progress for about 30 hours to a stage at which developing immature and mature spores can be visualized by immunofluorescence and the percentage of host cell infection can be calculated. When 1 or 10 million spores are used as the inocula, there was no significant difference in the percentage of infected host cells in CSA treated samples as compared to untreated samples (Figure 2.7B). However, when an inoculum of 50 or 100 million was used, there was a significant decrease in the percentage of infected host cells in the presence of the inhibitor. The reduction was 78% when 50 million spores were used and 68% when 100 million spores were used. These data therefore indicate a direct relationship between *E. intestinalis* spore adherence and host cell infection.

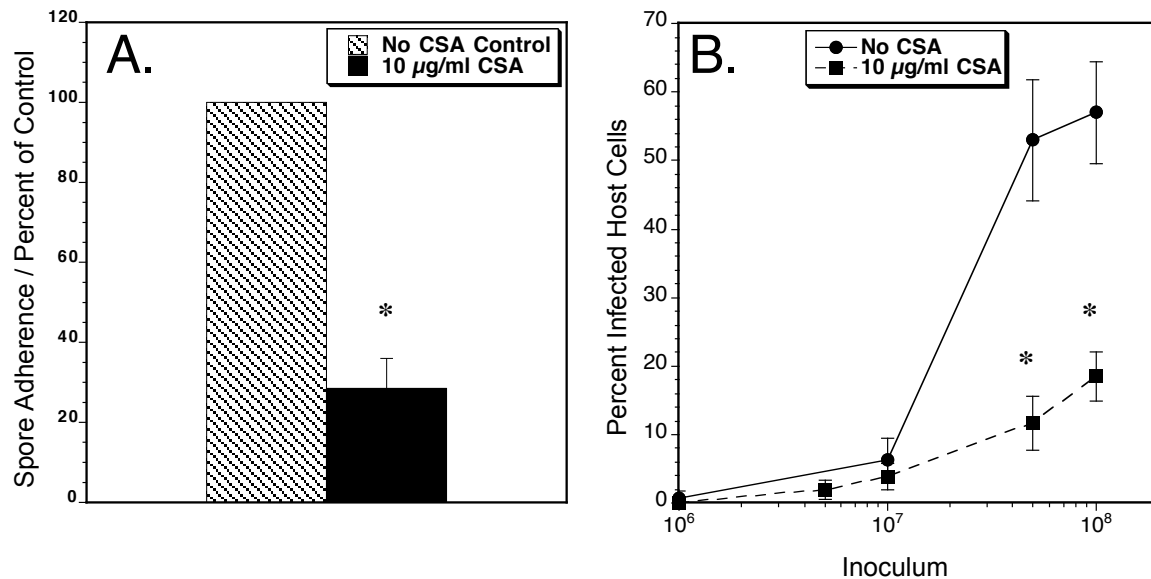


Figure 2.7 The effects of spore adherence inhibition on host cell infection. *E. intestinalis* spores were allowed to adhere to Vero cells monolayers with or without the addition of chondroitin sulfate A (CSA). (A) After four hours of incubation, one set of coverslips was removed to quantitate the percent inhibition. (B) The remaining coverslips were washed to remove unattached spores and placed back in culture for 30 hours. Infection was visualized by staining intracellular spore clusters with propidium iodide as described. The percentage of infected host cells was calculated by dividing the mean number of infected cells per field of magnification by the mean total number of cells per field of magnification. Twenty random fields were counted for each data point. The asterisk indicates significance at the $p < 0.0001$ level. This experiment was repeated three times with similar results.

Discussion

Our data indicate that microsporidia spore adherence to host cell surfaces involves a specific mechanism that is mediated by host cell surface sulfated GAGs. In these studies, all tested GAGs inhibited spore binding to host cell surfaces with the exception of non-sulfated dextran. This mechanism of adherence was confirmed by treating host cells with xylopyranoside to reduce the amount of surface GAGs, by using host cells deficient in surface GAGs, and by exposing host cells to sodium chlorate to reduce the level of GAG sulfation. In

each of these studies, *E. intestinalis* spore adherence to host cells was reduced compared to untreated or non-mutated host cells. Collectively, these data give strong evidence for the involvement of host cell surface sulfated proteoglycan in a specific spore adherence mechanism.

GAGs are found on the surface of almost all cell types (Bernfield et al. 1992; Yanagishita and Hascall 1992) and are used by many intracellular pathogens to attach and gain entry to host cells. For example, the capability of *Toxoplasma gondii* to infect a wide variety of hosts and tissues is thought to be due to its ability to recognize various host cell surface GAGs (Carruthers et al. 2000). Microsporidia are known to have a wide host range, and a variety of host cell lines can support *E. intestinalis* growth *in vitro* (Visvesvara et al. 1999). In our studies, *E. intestinalis* spore adherence was inhibited by five different GAGs. It is possible that microsporidia's ability to infect a wide range of hosts and tissues may correlate with its ability to use multiple GAGs for adherence. However, more research is necessary in order to conclusively establish this link.

Other intracellular microbial pathogens specifically use host cell surface heparan sulfate to attach and gain entry. The bacterial pathogens *Listeria monocytogenes* (Alvarez-Dominguez et al. 1997) and *Mycobacterium* spp. (Pethe et al. 2000) produce a surface heparin binding protein involved in adherence to epithelial cells. The Dengue (Chen et al. 1997) and foot-and-mouth viruses also interact with cell surface heparan sulfate, but these interactions alone may not in itself be sufficient for infection (Jackson et al. 1996). In addition, the parasites *Trypanosoma cruzi* (Herrera et al. 1994), *Plasmodium*

spp. (Frevert et al. 1993), and *Leishmania* spp. (Butcher et al. 1992; Love et al. 1993) have been reported to use heparan sulfate in host cell adhesions. Our data show that microsporidia spores apparently have a preference for sulfated heparan proteoglycans. The CHO mutant cell line deficient in heparan sulfate showed roughly the same reduction in spore adherence as the CHO mutant cell line deficient in both heparan sulfate and chondroitin sulfate. Moreover, the calculated EC₅₀ values indicate that heparin was the most efficient at spore adherence inhibition. These data indicate that even though a variety of GAGs can inhibit microsporidia spore adherence to host cells, microsporidia may preferentially use host cell heparan sulfate in adherence that is similar to that observed for other intracellular pathogens.

Another gastrointestinal pathogen, *Entamoeba histolytica*, recognizes the terminal galactose / N-acetyl-D-galactosamine residues of target glycoproteins that are found on both intestinal host cells and in colonic mucin (Petri et al. 1987). Invasive amebiasis initiates by attachment of the amoeba to the mucus layer followed by amebic adherence to mucosal epithelial cells (Petri and Mann 1993). Our data show that of the sulfated proteoglycans tested for microsporidia adherence inhibition, mucin may be the most physiologically relevant because the spores are produced and released into the intestine. A combination of mucin with a change in pH has been shown to induce the extrusion of the polar filament in spores in the absence cells (Pleshinger and Weidner 1985), but the conditions for spore activation *in vivo* are not known. It is possible the microsporidia may adhere in a similar manner by first attaching to intestinal mucus then gaining

access to the host cell surface. This method would allow microsporidia to circumvent the host cell protective measure by using intestinal mucus to its advantage.

Several viruses are known to use a combination of host cell receptors to attach and gain entry. For example, herpes viruses initially bind to host cell heparan sulfate via a viron glycoprotein (Feyzi et al. 1997; Geraghty et al. 1998). The virions then fuse with the host cell membrane using various other viral glycoproteins. Furthermore, the foot-and-mouth viruses use cell surface heparan sulfate to concentrate virus particles for subsequent integrin receptor binding (Jackson et al. 1996; Putnak et al. 1997). In this study, complete ablation of spore adherence to host cells is not achieved with the inhibitors tested. Depending on the inhibitor used, however, the maximal level of spore inhibition achieved is roughly 70% to 90% of control. One possible reason for this, other than not enough inhibitor used, is that there may be another mechanism of spore adherence that is independent of the sulfated proteoglycan mechanism. Additional studies are underway to determine if microsporidia use an additional mechanism for spore adherence to host cells.

Microsporidia spore adherence may be a host defense mechanism geared toward ridding the host of a potential infection. It has been shown that non-professional phagocytic cells, like those lining the intestines, can take up both live and dead *Encephalitozoon* ssp. spores through traditional phagocytic mechanisms involving host cell actin polymerization (Couzinet et al. 2000). These phagocytic mechanisms do not involve host cell membrane ruffling

characteristic of macropinocytosis (Couzinet et al. 2000; Foucault and Drancourt 2000). Once inside the host cell, the phagocytosed spores could be degraded. However, with the change in environmental conditions in degradation centers within host cells, it is possible that microsporidia spores could still activate and extrude its polar filament into the host cell cytoplasm and cause an infection (Couzinet et al. 2000). Alternatively, the internalized spores could theoretically pass through the host cell and emerge into the subepithelial layer. *Neisseria* use a similar mechanism to transverse epithelial barriers to enter the bloodstream (Gray-Owen 2003). In this scenario, spore adherence may be more relative to dissemination than infection. *Encephalitozoon* spp. and others are known to disseminate to other tissues and organs (Cali et al. 1993; Kotler and Orenstein 1999). However, further study is required to determine if there is a relationship between spore adherence and dissemination.

The functionality of microsporidial spore adherence is as of yet unknown. Spore germination is a process that requires multiple steps including spore activation, the build up of internal pressure, polar filament extrusion, and the passage of the infectious sporoplasm. Microsporidial spore adherence to host cell surfaces may be the initial event that signals the spore to activate and sets into motion the cascade of events leading to germination and infection. In support of this hypothesis, we clearly show that inhibition of spore adherence results in less host cell infection, indicating a direct association between spore adherence and infection. If spores are activated by contact with the host cell surface, it is postulated that a calcium influx may play an initial role in the

following cascade of events (Pleshinger and Weidner 1985; Leitch et al. 1993; He et al. 1996). On the other hand, if spore-cell contact is not signaling activation of the spore, the anchoring of a non-activated spore would be beneficial to the physical piercing of host cells by the polar tube when the spore becomes activated.

In summary, one specific mechanism of microsporidia spore adherence to host cells has been identified that involves host cell surface sulfated GAGs. Although these studies show that a variety of proteoglycans can inhibit spores from attaching to host cells, there appears to be a preference for heparan sulfate proteoglycans. These studies also show a direct association of spore adherence and host cell infection because reduced adherence due to exogenous proteoglycans in an *in vitro* assay results in reduced host cell infection. Identifying and characterizing the mechanism of spore adherence may lead to understanding of how spores become activated. This may ultimately lead to the development of novel therapeutics.

Acknowledgments: We thank Dr. Priscilla Wyrick and Ms. Judy Whittimore and the ETSU Transmission Electron Microscope Core Facility for the consultation and technical assistance for TEM, Dr. John Kalbfleisch for assistance with statistical analyses, and Dr. Sara Davis-Hayman for critical reading of the manuscript. This study was supported by Public Health Service grant AI055267 (to JRH) from the National Institute of Allergy and Infectious Diseases.

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CHAPTER 3

AUGMENTATION OF MICROSPORIDIA ADHERENCE AND HOST CELL INFECTION BY DIVALENT CATIONS

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Running Title: Microsporidia Adherence and Infection Augmentation by Divalent Cations.

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Southern, T. R., Jolly, C. E. and Russell Hayman, J. (2006). Augmentation of microsporidia adherence and host cell infection by divalent cations. *FEMS Microbiol Lett* 260(2): 143-9.

Abstract

The infection process of intracellular opportunistic microsporidia involves the forcible eversion of a coiled hollow polar filament that pierces the host cell membrane, allowing the passage of infectious sporoplasm into the host cell cytoplasm. Although the exact mechanism of spore activation leading to polar filament discharge is unknown, we have shown that spore adherence to host cells, which is mediated by sulfated glycosaminoglycans, may play a vital role. When adherence is inhibited, host cell infection decreases, indicating a direct link between adherence and infection. The goal of this study was to evaluate the effects of exogenous divalent cations on microsporidia spore adherence and infection. Data generated using an *in vitro* spore adherence assay show that spore adherence is augmented by manganese (Mn^{2+}) and magnesium (Mg^{2+}), but not by calcium (Ca^{2+}). However, each of the three divalent cations contributed to increased host cell infection when included in the assay. Finally, we show that Mn^{2+} and Mg^{2+} may activate a constituent on the microsporidia spore, not on the host cell, leading to higher infection efficiency. This report further supports recent evidence that spore adherence to the host cell surface is an important aspect of the microsporidial infection process.

Keywords: Adherence, Infectivity, Divalent Cation, Manganese, Magnesium, Calcium, *Encephalitozoon intestinalis*

Introduction

Microsporidia are obligate intracellular opportunistic pathogens that infect a wide range of hosts including insects, fish, birds, and mammals (Wasson and Peper 2000; Mathis et al. 2005). These organisms were once considered primitive eukaryotes but are now classified as highly derived fungal pathogens (Keeling et al. 2000; Keeling and Fast 2002). While approximately 1200 species of microsporidia have been identified, only 13 are known to infect humans. Of these, *Enterocytozoon bieneusi* is the most frequently encountered (Mathis et al. 2005). Immunocompromised individuals, particularly those with AIDS, are susceptible to *E. bieneusi* infection. Unfortunately, our understanding of *E. bieneusi* is limited due to the lack of a reliable *in vitro* culture system (Visvesvara et al. 1995). Like *E. bieneusi*, *Encephalitozoon* species are common causes of human microsporidiosis (Kotler and Orenstein 1999). *Encephalitozoon intestinalis* and *E. cuniculi* cause gastrointestinal disorders as well as a myriad of disseminated infections (Weber et al. 1994). Unlike *E. bieneusi*, *Encephalitozoon* species are easily cultured to high yields *in vitro* (Visvesvara 2002). A reliable culture system, coupled with the sequenced *E. cuniculi* genome, has contributed to a better understanding of these unique fungal pathogens and their interactions with host cells.

Microsporidia are unique in their infective process. Ingested spores release a hollow filament (or polar tube) that is used to transfer infectious sporoplasm into the host cell cytoplasm. Several hypotheses exist describing the method of polar tube entry into the host cell. One hypothesis suggests that the polar tube penetrates the host cell cytoplasmic membrane following forceful extrusion from the spore (Keohane and Weiss

1998). A second hypothesis suggests the extruded polar tube is internalized following interaction between polar tube proteins, particularly PTP1, and a yet unidentified host cell receptor (Xu et al. 2004). Regardless of the mechanism, infectious sporoplasm ultimately passes through the hollow tube and into the host cell cytoplasm where the organism begins to replicate. After development, the host cell ruptures releasing mature spores into the extracellular environment. For this mechanism of infection to be effective, it is essential for the spore to be in close proximity to the host cell. Otherwise, polar tube release would be misdirected and ineffective as a mode of infection.

In our previous studies, we have shown that microsporidia spores adhere to the surface of host cells via a mechanism involving host cell surface sulfated glycosaminoglycans (Hayman et al. 2005). Heparin, chondroitin sulfate A, and chondroitin sulfate B inhibited adherence as much as 88% when compared to control without exogenous glycans. The non-sulfated, negatively charged hyaluronic acid failed to inhibit adherence suggesting that adherence may not be directly linked to the negative charge. These data were confirmed by using compounds that either limited host cell surface expression of glycosaminoglycans or decreased surface sulfation. Furthermore, when spore adherence was inhibited by the addition of exogenous sulfated glycans, host cell infection was dramatically reduced. These results shaped our current hypothesis that spore adherence is an integral process of host cell infection.

In the current study, we further characterize spore adherence by examining the effects of divalent cations, which are documented effectors of numerous host-pathogen interactions. Examples include the Ca^{2+} dependent attachment of *Vibrio cholerae* to rabbit intestinal cells (Jones et al. 1976), the Ca^{2+} and Mg^{2+} modulated attachment of

Sporothrix schenckii to human fibronectin and endothelial cells (Lima et al. 2001; Figueiredo et al. 2004), and the inhibition of *Giardia intestinalis* adherence to intestinal epithelial cells by EDTA and EGTA (McCabe et al. 1991).

Historically, both cations and anions have been used to elicit microsporidia polar tube discharge *in vitro* (Keohane and Weiss 1998). Although cations and anions can passively enter the spore, it is suggested that only cations are responsible for germination (Frixione et al. 1994). In addition, calcium has been shown to play a significant role in spore germination. Chelation of Ca^{2+} with EGTA or the addition of calcium channel blockers reduce filament discharge (Leitch et al. 1993). And, by using a binding dye, calcium was localized to the polaroplast and was suggested to be intimately involved in polaroplast swelling leading to polar filament discharge (Weidner and Byrd 1982). Other cations such as potassium, sodium, magnesium, and barium had no apparent effect on polaroplast swelling. In this study, we provide evidence that cations are also involved in the augmentation of microsporidia spore adherence to host cells and this augmentation leads to an increase in host cell infection.

Materials and Methods

Microsporidia and host cell cultivation

African green monkey kidney cells (Vero; ATCC CCL-81) and rabbit kidney cells (RK-13; ATCC CCL-37) were used for cultivation of *E. intestinalis* or *E. cuniculi* spores as previously described (Hayman and Nash 1999; Hayman et al. 2001). Spores were purified from host cell debris by washing once with 0.25% sodium dodecyl sulfate followed by centrifugation and several washes with sterile H_2O . Spore stocks were

counted and stored in sterile H₂O at 4°C. Alternatively, microsporidia were purified by Percoll gradient centrifugation as previously described (Green et al. 1999). This protocol has the added advantage of separating mature and immature microsporidial forms.

Spore adherence assays

Vero and RK-13 were used as substrates for spore adherence assays as previously described (Hayman et al. 2005). Host cells were seeded on circular glass coverslips (18mm) at 5×10^5 in 12-well plates with normal growth medium and were allowed to grow to confluence. A serial dilution of MnCl₂, MgCl₂, or CaCl₂ from 1 mM to 0.001 mM was prepared in HEPES buffered saline (HBS) or fresh medium. Each divalent cation dilution was added to a well containing a host cell monolayer. In assays confirming the role of the divalent cation, 0.5 mM ethylenediaminetetraacetic acid (EDTA) was used for chelation. Ten million (1×10^7) *E. intestinalis* or spores were then added to each well followed by a 4 h incubation on ice. Our previous study showed no discernable difference in adherence between *E. intestinalis* and *E. cuniculi* spores (Hayman et al. 2005). These assays were performed on ice in an effort to minimize host cell surface protein recycling. Following the 4 h incubation, coverslips were washed thoroughly in phosphate buffered saline (PBS) to remove un-bound spores. Monolayers were fixed with acetone:methanol for 10 min at room temperature, and an immunofluorescence assay was performed to quantify the number of bound spores as previously described (Hayman et al. 2005). The results were expressed as the mean +/- standard deviation of 10-20 fields counted in a blinded fashion. Results were also

expressed as the percentage of adherent spores relative to control samples.

Significance was determined using the Student's *t* test.

Infectivity assays

Infectivity assays were conducted by first performing duplicate spore adherence assays in DMEM with divalent cation supplementation. While one set of coverslips was analyzed for adherence, the other set was washed of un-bound spores following adherence and placed in a clean 12-well plate with 1ml fresh medium per well without divalent cation. These monolayers were incubated for 36 hours at 37°C in 5% CO₂ to allow infection of host cells and development of microsporidia. Following incubation, coverslips were removed, thoroughly washed in PBS, and fixed in acetone:methanol. Coverslips were washed again in PBS followed by a 10 min incubation in 0.01% Uvitex 2B in PBS. The monolayers were again washed and inverted on a microscope slide. Microscopy using a UV filter was used to count the number of host cells and infected host cells per field. Ten to 20 fields were selected in a blinded fashion and evaluated at 400X magnification. The results were expressed as percentage of host cells infected.

Host cell and spore fixation

To determine which component is activated by the divalent cation, spore adherence assays were also conducted with fixed host cells or fixed spores. Initially, host cell monolayers were pretreated with 1 mM MnCl₂ in DMEM medium for 1 hour on ice. Control monolayers were incubated under the same conditions in DMEM medium with PBS. The host cell monolayers were then washed with PBS and fixed with cold 2%

paraformaldehyde for 15 min on ice. Fixed monolayers were washed thoroughly with PBS to remove residual fixative and were then used in spore adherence assays with or without post-fixation treatment with 1 mM MnCl_2 .

A similar series of assays was conducted with fixed *E. intestinalis* spores. Microsporidia spores were pelleted, suspended in PBS with 1 mM MnCl_2 , and incubated for 1 hour on ice. Control spores were incubated in PBS under similar conditions, but without exogenous MnCl_2 . All spores were washed 3 times with PBS and fixed with 2% paraformaldehyde overnight at 4°C. Spores were washed with PBS to remove residual paraformaldehyde. The fixed microsporidia spores were then used in spore adherence assays with live host cell monolayers in fresh medium with or without post-fixation treatment of 1 mM MnCl_2 .

Results

Mn^{2+} and Mg^{2+} , but not Ca^{2+} , augment spore adherence

To further characterize spore adherence, we examined the effects of the divalent cations Mn^{2+} , Mg^{2+} , and Ca^{2+} on this process. When added independently to the adherence assay conducted in HBS, the cations from MnCl_2 and MgCl_2 augmented spore adherence to host cell surfaces in a dose-dependant manner (Figure 3.1). No augmentation of spore adherence was detectable at the minimum concentration of divalent cation evaluated (0.001 mM). However, MnCl_2 augmented spore adherence 3-fold at a concentration of 0.1 mM. At 1 mM, both MnCl_2 and MgCl_2 augmented spore adherence greater than 4-fold. At 10 mM, MnCl_2 and MgCl_2 augmented spore

adherence by >6-fold and >5-fold, respectively. Interestingly, the addition of CaCl_2 did not influence spore adherence at any concentration assayed.

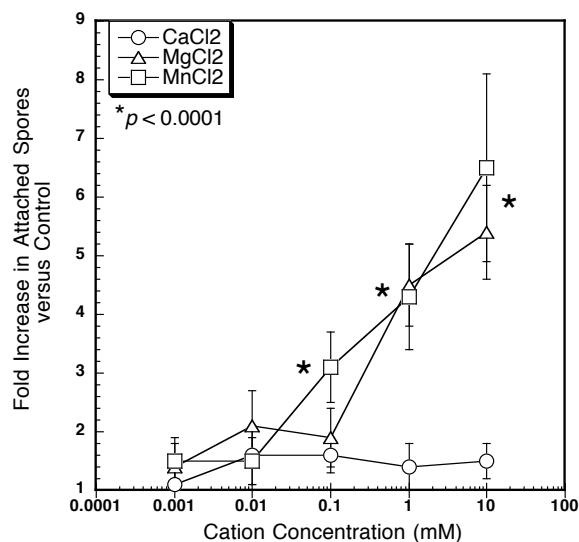


Figure 3.1 Effects of divalent cations on spore adherence. Spore adherence assays were conducted with *E. intestinalis* spores in HEPES buffered saline with serial dilutions of MnCl_2 , MgCl_2 , or CaCl_2 to evaluate the activity of each divalent cation across a broad range of physiologically relevant concentrations (0.001mM–10mM). PBS was used as control. The data shown are from one experiment, which was repeated three times with similar results. Asterisks indicate a statistical difference from control as determined by the Student's *t*-test ($p < 0.0001$).

To confirm these findings, the adherence assay was performed in the presence of both the cation and the chelator EDTA. The addition of 0.5 mM EDTA is sufficient to completely abrogate the augmentation seen with 1 mM concentrations of both MnCl_2 and MgCl_2 (Figure 3.2). Addition of the chelator reduces spore adherence to basal levels similar to the amount of spore adherence observed when exogenous divalent cations are not present. The addition of EDTA to assays with CaCl_2 had no effect on spore adherence.

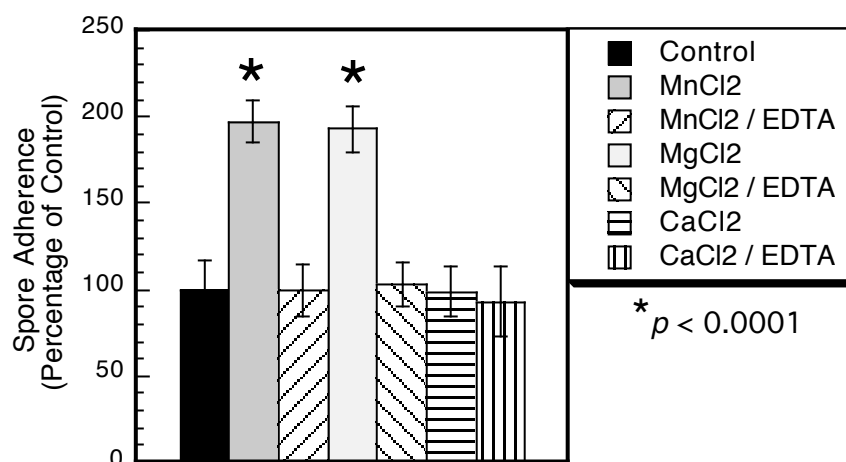


Figure 3.2 Effects of EDTA on divalent cation augmented spore adherence. Spore adherence assays were conducted with *E. intestinalis* spores with or without the addition of 1mM MnCl₂, MgCl₂, or CaCl₂. In addition, 0.5 mM EDTA was added to chelate the cations. The adherence assay was conducted as described. The data shown are from one experiment, which was repeated three times with similar results. Asterisks indicate statistical difference from control as determined by the Student's *t*-test ($p < 0.0001$).

Mn²⁺, Mg²⁺, and Ca²⁺ increase host cell infection efficiency

To confirm that the addition of divalent cations had an effect on host infection, infectivity assays were conducted with each divalent cation at a 1 mM final concentration in the presence or absence of the chelator EDTA (Figure 3.3). After the adherence assay was performed and the un-bound spores were removed, the host cells were cultured for an additional 36 hours, a time at which the level of host cell infection could be quantified by Uvitex-2B staining. These data indicate that when included in the spore adherence portion of the assay, each of the divalent cations caused increased host cell infection. MnCl₂, MgCl₂, and CaCl₂ (1 mM) each facilitated an approximate 2-fold increase in infectivity. The increased infectivity attributed to MnCl₂ and MgCl₂ is not surprising because both divalent cations also augment spore adherence. In addition, when EDTA is added, the level of host cell infection is reduced to control levels,

suggesting that the observed increase in spore adherence is due to the divalent cation and is not an artifact of the *in vitro* assay system. These data further support a direct correlation between spore adherence and host cell infection.

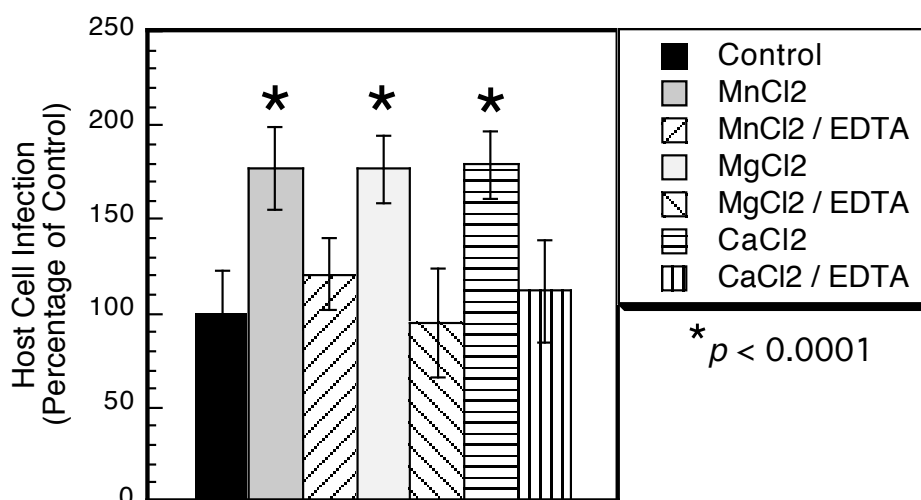


Figure 3.3 Effects of EDTA on divalent cation augmented infectivity. Infectivity assays were conducted with *E. intestinalis* spores with or without the addition of 1mM MnCl₂, MgCl₂, or CaCl₂. In addition, 0.5 mM EDTA was added to chelate the cations. The infectivity assay was conducted as described. The data shown are from one experiment, which was repeated three times with similar results. Asterisks indicate statistical difference from control as determined by the Student's *t*-test ($p < 0.0001$).

Interestingly, CaCl₂ does not augment spore adherence (Figure 3.2) but does facilitate an increase in host cell infection (Figure 3.3). Calcium-induced spore activation leading to polar filament discharge has been documented (Weidner and Byrd 1982; Pleshinger and Weidner 1985; Leitch et al. 1993). CaCl₂, along with an increase in pH, promote polar filament discharge, whereas, EGTA, calcium channel antagonist, and calmodulin inhibitors prevent discharge (Pleshinger and Weidner 1985; Leitch et al. 1993). It has been speculated that the movement of calcium from polaroplast membranes leads to a swelling of the polaroplast and polar filament discharge (Weidner

and Byrd 1982). The fact that calcium does not augment adherence but is integrally involved in polar filament discharge is evidence that adherence and activation are two separate events.

Contrasting immature and mature spore adherence and infection

E. intestinalis and *E. cuniculi* spores develop in an asynchronous fashion (Cali et al. 1993). Upon host cell rupture, noninfectious immature spores (meronts, sporonts, and sporoblasts) may be released along with infectious mature spores. To determine whether the maturity of spores affects adherence and infectivity, immature spores were separated from mature spores by gradient centrifugation, and the adherence assay was performed with both spore populations in the presence or absence of 1 mM MnCl_2 (Figure 3.4A). As expected, the addition of the divalent cation augmented spore adherence of mature spores approximately 3-fold. Moreover, adherence augmentation of the immature spores was more than 7-fold. Interestingly, there was no significant difference in the total number of bound spores between immature and mature spores in the presence of Mn^{2+} .

Following the adherence assay, un-bound spores were removed and host cell infection was allowed to progress (Figure 3.4B). Compared to the mature spores, the immature spores were essentially non-infectious. It is possible that the limited percentage of infected host cells detected with the immature spore inoculum was due to contaminating mature spores from the gradient centrifugation process. With mature spores, the percentage of infected host cells was greatly enhanced by the addition of MnCl_2 . The absence of divalent cation during adherence resulted in 16% host cell

infection, compared to 48% in the presence of 1 mM MnCl_2 . These data show that while the immature spores do not have the ability to infect host cells, they are capable of adhering to host cells in a manner similar to that of mature spores. At this time, the fate of immature, non-infectious spores on the surface of host cells is unknown. One might speculate that these spores are, perhaps, phagocytized and degraded by the host cell. However, it has been shown that apical phagocytosis by host cells is not a significant event (Leitch et al. 2005).

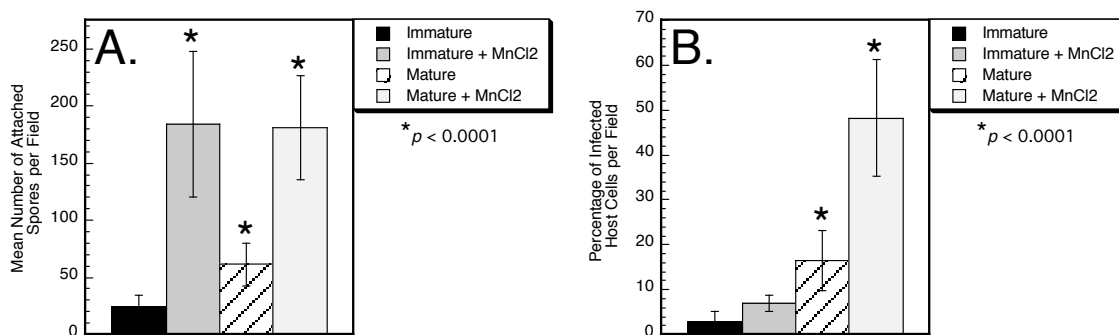


Figure 3.4 Effects of MnCl_2 on immature and mature spore adherence and infection. Spore adherence assays (A) and infectivity assays (B) were conducted with both immature and mature *E. intestinalis* spores in the presence or absence of 1 mM MnCl_2 as described. Immature spores were separated from mature spores by gradient centrifugation. The data shown are from one experiment, which was repeated three times with similar results. Asterisks indicate statistical difference from control as determined by Student's *t*-test ($p < 0.0001$).

Divalent cations affect a putative spore ligand

To determine if Mn^{2+} acts upon the host cells or the spores, these components were treated with a paraformaldehyde fixative either prior to or following divalent cation activation. Pre-treating host cells with 1 mM MnCl_2 and subsequently replacing the medium with medium containing no exogenous MnCl_2 did not augment adherence (Figure 3.5A). However, the addition of 1 mM MnCl_2 with spores in the assay without

host cell fixation significantly increased adherence, as expected. Interestingly, treating the host cells with the fixative did not significantly alter spore adherence. Roughly the same number of spores bound live cells as cells treated with the fixative. Incubating host cells with MnCl_2 prior to fixation did not augment adherence. However, MnCl_2 addition along with spores following fixation did result in adherence augmentation.

The fact that MnCl_2 could augment spore adherence when host cells were fixed strongly suggests that the cation is activating a ligand on the spore surface. To confirm these data, the paraformaldehyde fixative was used to fix the spores in the “active” state following MnCl_2 treatment (Figure 3.5B). Although the fixative solution alone, without the addition of divalent cations, reduced the level of spore adherence to approximately 63% of the unfixed control spores, adherence augmentation occurred when the spores were induced to an active state with MnCl_2 treatment prior to fixation. Adherence was not augmented if MnCl_2 was added after fixation.

These two experiments indicate that microsporidial spores have a surface molecule that may be activated by specific divalent cations leading to increased spore adherence. The identity of this spore adherence ligand is currently unknown. Because adherence is, in part, activated by divalent cations and because it is possible that the activated protein is binding host cell glycoproteins (Hayman et al. 2005), this candidate adherence ligand may in fact be a lectin.

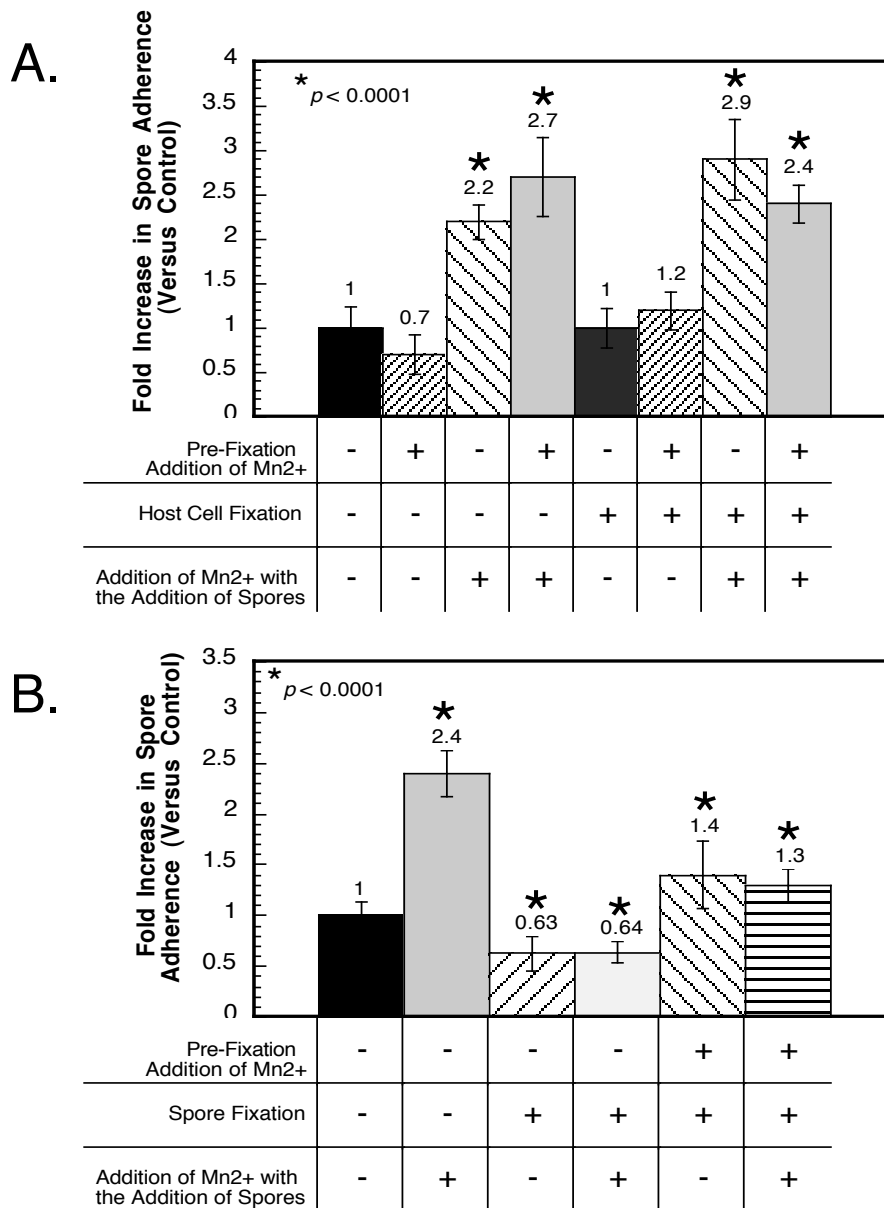


Figure 3.5 MnCl_2 augments spore adherence by activating a putative protein(s) on the spore. (A) A series of spore adherence assays was conducted to determine if the divalent cation Mn^{2+} activates a host cell constituent leading to augmentation of spore adherence. Both fixed and live host cell monolayers were used in the spore adherence assay with or without the addition of 1 mM MnCl_2 . (B) A series of spore adherence assays were conducted to determine if the divalent cation Mn^{2+} activates a spore constituent leading to augmentation of spore adherence. This series of assays used *E. intestinalis* spores that were either pretreated with 1mM MnCl_2 in PBS or were incubated in PBS alone. Fixed and live spores were used in spore adherence assays with or without the addition of 1 mM MnCl_2 at the time of assay. The data are presented as the fold difference in spore adherence compared to non- MnCl_2 treated, non-fixed controls and are from one experiment, which was repeated three times with similar results. Asterisks indicate statistical difference from control as determined by the Student's *t*-test ($p < 0.0001$).

Discussion

To date, five proteins have been identified in the spore wall external to the spore plasma membrane. Two spore wall proteins (SWP1 and SWP2) have been localized to the exospore region in members of the Encephalitozoonidae family (Bohne et al. 2000; Hayman et al. 2001). These proteins have conserved cysteine residues in their N-terminal domains, indicating similar functions, but the C-terminal domains differ. SWP1 has a glycine/serine repetitive element, and SWP2, which to date has been identified only in *E. intestinalis*, has fifty 12-15 amino acid repetitive units of unknown function (Hayman et al. 2001). Recently, three additional proteins have been localized to the chitinous endospore region, which is located between the exospore region and the plasma membrane (Peuvel-Fanget et al. 2005; Xu et al. 2006). One protein, called endospore protein 1 (EnP1), was identified by immunoscreening of a cDNA library (Peuvel-Fanget et al. 2005). It is cysteine rich and is postulated to be involved in spore wall assembly by disulfide bridging. Another protein, identified by two separate groups, is called EnP2 or SWP3 (Peuvel-Fanget et al. 2005; Xu et al. 2006). This 20-22 kDa protein is predicted to be O-glycosylated and has the sequence motifs necessary for glycosylphosphatidylinositol (GPI)-anchoring. The third protein, a putative chitin deacetylase (called EcCDA), is present in two isoforms of 33 and 55 kDa and is associated with the plasma membrane of developing spores (Brosson et al. 2005). Although it is possible that any of these proteins could be responsible for the divalent cation enhanced adherence of spores, it is expected that this adherence ligand would be accessible in the exospore region. Whether SWP1 or SWP2 is capable of such activity remains to be determined.

Spore adherence to host cells may serve as the initial event that signals the beginning of the infection process. Historically, the events of polar filament discharge and host cell infection have been divided into four steps: 1) activation, 2) increased internal osmotic pressure, 3) polar filament release by eversion, and 4) the movement of sporoplasm from the spore through the polar tube and into the host cell cytoplasm. Because our findings confirm earlier studies showing that calcium plays an integral role in activation but is not involved in spore adherence augmentation to host cells, we propose that spore adherence is an event that precedes activation. Our hypothesis is supported by the fact that spores are commonly found attached to host cell surfaces with intact, non-discharged, polar filaments (Hayman et al. 2005). Following adherence, it is possible that the physical joining of the spore to the host cell surface may initiate a signaling cascade resulting in the activation step. Previously, we have shown that adherence is directly linked to infection because inhibiting adherence reduces infection. In this study, we show that by augmenting adherence, host cell infection increases. Together, these two studies substantiate the direct relationship between adherence and infection. Finally, this study indicates that microsporidia spores have a surface molecule, perhaps a lectin, which can be activated in the presence of magnesium or manganese divalent cations. Once activated, spores adhere more efficiently to host cell surfaces, which leads to more efficient infection. Examining and gaining an understanding of the mechanisms of spore adherence as it may relate to activation may ultimately lead to the development of novel therapeutics.

Acknowledgments: We thank S.R. Davis-Hayman for critical reading of the manuscript. This work was supported by grant AI055267 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

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CHAPTER 4

IDENTIFICATION AND CHARACTERIZATION OF THE *ENCEPHALITOZOON CUNICULI* MICROSPORIDIA ADHERENCE PROTEIN (EcMsAP) AND ITS INVOLVEMENT IN SPORE ADHERENCE TO HOST CELLS *IN VITRO*

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Running Title: Identification and characterization of the microsporidia adherence protein

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Abstract

Microsporidia are spore-forming fungal pathogens that require the intracellular environment of a host cell for propagation. Human microsporidiosis is typically limited to immunocompromised individuals and is characterized by severe diarrhea with potential for wasting disease and disseminated infections. While the infection process is well characterized, little is known about the molecular events that govern spore adherence and infection initiation. The goal of this study was to examine the physical interaction between the microsporidia spore wall and the host cell surface during spore adherence. In this study we identify a single *Encephalitozoon cuniculi* spore protein that interacts with the host cell surface. The *E. cuniculi* protein was identified as ECU01_0820, a hypothetical protein with no known function. Analysis of the ECU01_0820 amino acid sequence revealed two heparin-binding motifs and an integrin-binding motif. These highly conserved sequences are characteristic of proteins that bind constituents on the cell surface or extracellular matrix. An *E. intestinalis* homologue of the ECU01_0820 gene was also identified using a subtractive cDNA library. Sequence analysis revealed three heparin-binding motifs and an integrin binding motif at the conserved location. The ECU01_0820 gene was cloned and recombinant protein was produced for antibody production. Immunotransmission electron microscopy using ECU01_0820 antibodies revealed that the ECU01_0820 protein is embedded in the microsporidial endospore, exospore, plasma membrane, and is found in high concentration on the anchoring disk. Recombinant ECU01_0820 protein and protein A/G purified

ECU01_0820 antibodies also inhibited spore adherence to host cells when used in spore adherence assays *in vitro*. Site-directed mutagenesis of the heparin-binding motifs of the ECU01_0820 gene dramatically influenced the ability of the recombinant protein to inhibit spore adherence and infection. Collectively, these data suggest that ECU01_0820, known here as *Encephalitozoon cuniculi* Microsporidia Adherence Protein (MsAP), interacts with the host cell surface and potentially plays a role in microsporidia spore adherence to host cells.

Key words: Microsporidia, *Encephalitozoon cuniculi*, Microsporidia Adherence Protein, Adherence, Infectivity

Introduction

Microsporidia are obligate intracellular opportunistic organisms that are currently classified as highly derived fungal pathogens (Keeling and Fast 2002; Keeling 2003; Gill and Fast 2006). To date, more than 1200 species of microsporidia belonging to almost 150 genera are known to science.

Microsporidia infect a wide variety of hosts including insects, fish, and mammals, including humans. Most human infections are attributed to 13 species of microsporidia that belong to 8 genera including *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Brachiola*, *Pleistophora*, *Trachipleistophora*, *Vittaforma*, and *Microsporidium*.

Human microsporidiosis is most often attributed to *Enterocytozoon bieneusi*, although *Encephalitozoon* species are also commonly diagnosed. Until recently, human microsporidiosis was almost exclusively diagnosed from individuals with AIDS (Lambl et al. 1996). Improved detection and better surveillance have contributed to an increase in observed microsporidiosis in other immunocompromised populations including transplant recipients and individuals taking immunosuppressive therapies. Although rare, cases of microsporidiosis in otherwise healthy individuals have also been reported (Weber et al. 1994; Fournier et al. 1998; Mathis et al. 2005).

Human microsporidiosis is typically characterized by voluminous diarrhea, although severe cases may result in wasting disease or disseminated infections (Canning and Hollister 1992). Otherwise healthy individuals exposed to microsporidia typically show no symptoms of infection but may exhibit

unexplained self-limiting diarrhea (Weber et al. 1994). The most common treatment for microsporidiosis is albendazole, a broad-spectrum antiprotozoal benzimidazole that disrupts microtubule polymerization in the developing parasite. A second treatment, fumagillin, is used topically to treat ocular microsporidiosis, but may also be used systemically (Didier 1997). While albendazole and fumagillin are used to treat some forms of microsporidiosis, they cannot be used as a broad-spectrum treatment for all microsporidial infections. Furthermore, toxicity of fumagillin limits its use as an effective systemic anti-microsporidial therapy (Didier et al. 2006).

Our ability to identify new therapies for treating and preventing microsporidiosis is largely dependent upon our understanding of the molecular mechanisms that govern host cell recognition and the initiation of infection. Unfortunately, little is known about these parasite-host interactions. It is known, however, that microsporidia spores adhere to glycosaminoglycans (GAGs) on *in vitro* grown host cells (Hayman et al. 2005; Leitch et al. 2005). Inhibition of GAG mediated spore adherence ultimately reduces infection while augmentation of adherence by specific divalent cations increases infection (Hayman et al. 2005; Southern et al. 2006).

The goal of this study was to further evaluate microsporidia spore adherence by examining the physical interactions at the spore-host cell interface. A single ~40kDa microsporidial protein, known here as *Encephalitozoon cuniculi* Microsporidial Attachment Protein (EcMsAP), was identified as a spore wall associated protein that interacts with the host cell surface. Data overwhelmingly

support the role of EcMsAP in spore adherence and indicate that MsAP may be a potential target for the development therapeutics to treat or even prevent microsporidiosis.

Materials and Methods

Microsporidia and Host Cell Cultivation

African green monkey kidney cells (Vero; ATCC CCL-81) and rabbit kidney cells (RK-13; ATCC CCL-37) were used for cultivation of microsporidia spores. Adherent cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, Md.) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml), and 2% fetal bovine serum (BioWhittaker) in 5% CO₂ at 37°C (Hayman and Nash 1999). For microsporidial spore propagation, confluent host cell monolayers were grown in 75-cm² flasks and infected with spores as previously described (Hayman *et al.*, 2001). Infected cells were monitored for 12-14 days with medium replacement every 2-3 days. Spores were harvested periodically until most host cells were dead. The spores were purified from host cell debris by washing once with 0.25% sodium dodecyl sulfate followed by centrifugation and several washes with sterile H₂O. Spores were washed until free of host cell debris. Spore stocks were then counted using a hemacytometer and stored at 4°C.

Host cell-binding assay, SDS-PAGE, and Western Analysis

A host cell-binding assay was designed to determine which, if any, microsporidia spore proteins interact with the host cell surface. Ten billion (10^9) *E. cuniculi* spores were labeled with NHS-biotin according to the manufacturers recommendations (Pierce). Spores were suspended in 100 μ l of SDS-boiling buffer (120 mM Tris-HCl pH 7.5, 5% SDS, and 100 mM DTT). Spores were boiled for 10 minutes followed by incubation at room temperature for 30 minutes. The spore suspension was centrifuged for 10 minutes at 13,000xg at 4°C. The supernatant was moved to a clean microcentrifuge tube and deoxycholic acid was added to 0.02% final concentration. The sample was incubated for 30 minutes at room temperature followed by addition of trichloroacetic acid (TCA) to a final concentration of 20%. The protein sample was precipitated overnight at 4°C with rocking. The sample was centrifuged at 18,000xg for 20 minutes and the supernatant was removed. The protein pellet was incubated in ice-cold acetone for 30 minutes at 4°C followed by a second centrifugation at 18,000xg. The supernatant was removed and the pellet was air dried. The protein pellet was solubilized in 200 μ l of host cell binding buffer (120 mM Tris-HCl pH 6.8 with 1% glycerol) at stored at -20°C until needed. Vero monolayers were grown on 18 mm glass coverslips in a 12-well plate according to the conditions above. Once confluent, a single monolayer was washed in sterile PBS (x3) and the TCA precipitated protein sample was incubated on the Vero monolayer for 2 hours at room temperature. The Vero monolayer was then washed in sterile PBS (x3) to remove non-bound spore proteins. The monolayer was solubilized in 5% SDS

with 100 mM DTT and boiled for 10 minutes. The solubilized protein sample was resolved by SDS-PAGE and the protein was transferred to nitrocellulose for Western analysis. The nitrocellulose blot was blocked in 5% non-fat dry milk for 60 minutes followed by 3 washes in TBS-T. The blot was incubated for 1 hour in streptavidin conjugated to alkaline phosphatase (1:2000 in TBST) followed by 3 washes in TBST. Proteins were detected using NBT/BCIP (Pierce).

The host cell-binding assay was also conducted with recombinant EcMsAP. The recombinant protein was expressed and purified by nickel affinity chromatography (see below). The protein solution was dialyzed against 100 mM tris buffer (pH 6.8). Protein precipitated during dialysis was collected by centrifugation and solubilized overnight in host cell binding buffer at 95°C. The sample was centrifuged and the supernatant was stored at -20°C until needed.

MALDITOF-MS Analysis

The Coomassie stained gel band corresponding to the molecular weight of interest (~40 kDa) from the host cell-binding assay was excised and submitted for mass spectrometry and database analysis. Sample preparation, mass spectrometry (MALDITOF-MS), and database analysis were performed by Midwest BioServices (Overland, KS).

Cloning and Recombinant Protein Expression

The gene encoding EcMsAP was PCR amplified directly from *E. cuniculi* genomic DNA using primers EcCD22-E (5'-GGAATTCAAGGCTCTTCACCTTAC

AGG-3') and EcCD22-X (5'-GTACTCGAGATCGAGATCGAGAGGTCCAA-3'). PCR product size was evaluated by 0.7% agarose gel electrophoresis. The remaining PCR product was cleaned (Qiagen), digested with the appropriate restriction enzymes, and cloned into the pET21 vector (Novagene). XL1-Blue *E. coli* cells (Stratagene) were transformed with the pET21a vector containing the EcMsAP gene. The plasmid was recovered for sequencing and transformation into *E. coli* Rosetta Gami (RG) expression cells (Novagene). A clonal population of transformed RG cells was grown in LB to an OD₆₀₀ 0.6. The culture was induced with IPTG (1 mM final concentration) and incubated at 37°C shaking for 4 hrs. Bacteria were harvested by centrifugation, suspended in 500 ul PBS, and sonicated for 90 seconds. The sonicated sample was centrifuged and the soluble fraction was removed to a clean microcentrifuge tube. The remaining insoluble fraction was suspended in 250 ul 5% SDS with 2% β-mercaptoethanol followed by sonication for 60 seconds. The SDS treated sample was centrifuged to remove cell debris. A non-induced bacterial control sample was also processed as described. Soluble and insoluble fractions were run in duplicate on a 4-12% Bis-Tris polyacrylamide gel. One half of the gel was Coomassie stained and the other half was used for Western Analysis. Protein transferred to nitrocellulose was blocked using 5% non-fat dry milk for 30 minutes. The blot was washed in tris buffered saline with 0.05% Tween-20 (TBS-T) followed by incubation in a monoclonal HIS-tag antibody (1:2000 in TBS-T; Sigma). The blot was washed in TBST followed by incubation in a rabbit anti-mouse alkaline

phosphatase conjugated secondary (1:2000 in TBS-T for 1 hr). The blot was washed in TBST and protein was detected using NBT/BCIP (Pierce).

Histidine-tagged recombinant protein was expressed in RG cells as described above. Following protein expression, bacteria were centrifuged (10 min at 3700xg) and the pellet was suspended in 25ml 1x phosphate buffer. The bacteria suspension was sonicated (power level 15; Microson Ultrasonic Cell Disruptor) for 30 seconds (x3). The suspension was centrifuged (10 min at 3700xg) and the pellet was suspended in 35 ml binding buffer (8M urea in 1x phosphate buffer with 20 mM imidazole) followed by sonication at power level 15 (Microson Ultrasonic Cell Disruptor) for 30 seconds (x3). The sample was solubilized overnight at 4°C rocking followed by centrifugation (25 min at 10,000xg). The supernatant was applied to at equilibrated 5 ml nickel affinity column (GE Biosciences). The column was washed with 5 column volumes of Binding Buffer followed by 3 column volumes of elution buffer (8M urea in 1x phosphate buffer with 300 mM imidazole). Ten 1.5 ml elution fractions were taken and a portion of each sample was resolved using SDS-PAGE. Protein containing fractions were dialyzed against 10 mM Tris buffer (pH 7.4) with 0.5 mM EDTA. The resulting dialysate was centrifuged and the protein containing supernatants were pooled and stored at -20°C for further use. Precipitated protein was also collected at stored at -20°C for further use.

Antibody Production and Protein A/G Purification

Serum from candidate rabbits was screened using Western analysis to detect prior exposure to microsporidia. Naive rabbits were selected for antibody production with the recombinant EcMsAP protein (ProteinTech Group, Inc.). Following antibody production, pre-bleed and final bleed sera were Protein A/G purified according to the manufacturers recommendations (Pierce). Briefly, a gravity flow column containing 250 ul Protein A/G agarose was poured and the column was equilibrated with binding buffer. The serum sample was applied to the column followed by thorough washing with binding buffer. The purified antibodies were eluted from the column with elution buffer and the pH of the eluate was adjusted to neutral with 100 ul of neutralization buffer. Purified antibodies were stored at -20°C until needed.

Immuno-Transmission Electron Microscopy

Confluent RK-13 monolayers grown in T-75 cm² flasks were infected with *Encephalitozoon cuniculi* or *E. intestinalis* spores. Infected monolayers were maintained for at least 3 days or until numerous infected cells were visible by light microscopy. Heavily infected monolayers were fixed for 1 hour with 2% paraformaldehyde and 0.05% glutaraldehyde in 0.2 M Sorenson's buffer. Monolayers were then washed thoroughly in 0.1 M Sorenson's buffer and scrapped from the flask. The host cell debris was enrobed in 3% SeaKem agar and the enrobed pellet was washed three times in 0.1M Sorenson's buffer at 4°C for 15 minutes. The enrobed pellet was dehydrated in 35%, 50%, and 70%

methanol at 4°C for 5 minutes at each dilution. The pellet was incubated in 90% methanol for 30 minutes at -20°C. The pellet was then sequentially incubated in following solutions at -20°C for 1 hour: Lowicryl K4M resin:90% methanol at 1:1, Lowicryl K4M resin:90% methanol at 2:1, and Lowicryl K4M resin only. The pellet was then incubated in Lowicryl K4M overnight at -20°C. The pellet was then imbedded in Lowicryl K4M resin with photopolymerization at -20°C for 2 days, 4°C for 2 days, and at room temperature for 2 days. Ultrathin sections of the Lowicryl embedded pellet were applied to gold grids. The sections were blocked for 5 minutes in 1% albumin and 0.01M glycine prepared in PBS. The sections were then incubated for 40 minutes at 37°C in a 1:25 dilution of protein A/G purified polyclonal rabbit antisera generated against recombinant EcMsAP. The protein A/G purified antibodies were diluted in the albumin/glycine blocking solution. Sections were blocked again for 5 minutes followed by incubation in a 1:200 dilution of AuroProbe EM 15 nm gold-labeled goat anti-rabbit IgG (H+L). Sections were washed 3 times in PBS for 5 minutes each followed by 3 fifteen-minute washes in sterile water. Finally, sections were counterstained with 5% uranyl acetate followed by a sterile water rinse. Sections were viewed using a Tecnai 10 (FEI) transmission electron microscope.

Site-Directed Mutagenesis

Heparin-binding motifs were sequentially deleted from the parent EcMsAP gene previously cloned into the pET21a vector. Site-directed mutagenesis was conducted using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene)

according to manufacturers recommendations. Briefly, primer sets were designed to delete heparin-binding motif #1 (5'-GCATCGAGCCGGTTGAGATCGTCGTCAATCCATC-3' and 5'-GATGGATTGACGACGATCTGTTGGCCGAGCTACG-3', heparin-binding motif #2 (5'-TACAGAAACCTCCACCACAAGCTCCGAGAGCTT-3' and 5'-AAGCTCGCGGAGCTTGTGGTGGAGGTTTCTGTA-3'), and an N-terminal portion of the EcMsAP gene (5'-TACCTCCAGGCAATGGTGCTCTACTGGAA-3' and 5'-TTCCAGTAGAGCACCATTGCCTGGAGGTA-3'). The N-terminal deletion was conducted to determine if amino acid deletion unduly influences protein activity during *in vitro* assays. The parent EcMsAP construct in the pET21 vector was used as the template for site directed mutagenesis. Once each deletion mutant was created, the PCR products were treated with DpnI to digest the methylated template DNA leaving only the mutated construct. The mutant DNA constructs were gel purified and sequenced to verify mutagenesis. Four mutant constructs were produced using this method including Heparin-binding motif #1 Deletion Mutant (HBM1DM), Heparin-binding motif #2 Deletion Mutant (HBM2DM), Double Deletion Mutant (DDM), and the control N-Terminal Deletion Mutant (NTDM). Each mutant protein was expressed and purified as described above.

Spore Adherence Assays

Vero cells (1×10^5) were seeded on circular glass coverslips (18mm) in 12-well plates with normal growth medium and were allowed to grow to 95% confluence. Serial dilutions of recombinant protein (1 ug/ml to 0.001ug/ml) were

made in 2% DMEM supplemented with L-glutamine, antibiotics, and FBS. Old medium was removed from each well and 1 ml of fresh medium with recombinant protein was added. The 12-well plate was incubated on ice for 1 hr during. Ten million (10^7) *E. intestinalis* or *E. cuniculi* spores were then added to each well followed by a 4 hr incubation on ice. Assays were performed on ice to minimize host cell surface protein recycling and phagocytosis. Following the 4 hr incubation, coverslips were washed thoroughly in sterile PBS to remove non-bound spores. Monolayers were fixed with acetone:methanol for 10 min at room temperature and an immunofluorescence assay was performed. Monolayers were incubated in 1 ml blocking buffer (5% FBS in PBS) for 1 hour at room temperature. Rabbit antiserum raised against *E. cuniculi* spores was used as the primary (1:1,000 in blocking buffer) and a fluorescein isothiocyanate-conjugated (FITC) anti-rabbit immunoglobulin was used as secondary (1:500 in blocking buffer; Rockland Immunochemicals). The rabbit antiserum was also cross-reactive with *E. intestinalis* spores. Fluorescence microscopy (Zeiss; Axiovert S100) was used to count the number of bound spores per field of magnification at 630X. Results are expressed as the mean \pm standard deviation of 20 fields counted in a blinded fashion. Results may also be expressed as the percentage of adherent spores relative to control samples. Statistical significance was determined using the Student's t-test.

Host Cell Infection Assays

Host cell infection assays were conducted by first conducting the spore adherence assay in duplicate. Following the spore adherence assay, one set of coverslips were washed, fixed, and prepared for fluorescence microscopy and spore adherence quantification. The duplicate set of coverslips was washed of non-bound spores and placed in a clean 12-well plate with 1ml fresh 2% DMEM supplemented with fetal bovine serum, antibiotics, non-essential amino acids, and L-glutamine. These monolayers were incubated for 36 hours at 37°C in 5% CO₂ to allow infection of host cells and development of vacuole-bound microsporidia. Following incubation, coverslips were washed in PBS and host cells were fixed in acetone:methanol for 10 min at room temperature. Coverslips were washed again in PBS followed by incubation in 0.01% Uvitex for 10 min at room temperature. The monolayers were washed with PBS and inverted on a microscope slide. The coverslips were sealed with cement and UV microscopy (Zeiss; Axiovert S100) was used to evaluate infection at 400X. Host cells and infected host cells were counted in 10-20 randomly selected fields. Results are expressed as a percent of host cells infected. Statistical difference was determined using the Student's t-test.

Biomolecular Interactions Analysis

Biomolecular interactions analysis was conducted using a BIAcore 2000 instrument. All reactions were conducted at 4°C unless otherwise specified. To activate the carboxymethylated dextran on the sensorchip surface, EDC (0.1 M)

and NHS (0.4 M) were injected at a flowrate of 5 ul/min for 15 minutes. Two micrograms of tetra-HIS monoclonal antibody were suspended in 100 ul of a 100 mM sodium acetate solution (pH 4.0). Following activation of the CM5 sensorchip surface, the monoclonal antibody solution was injected at a flow rate of 5 ul/minute for 10 minutes. Active sites not occupied by the tetra-HIS monoclonal antibody were blocked with 1 M ethanolamine (pH 8.5) injected at a flow rate of 5 ul/minute for 20 minutes. The chip surface was then equilibrated with HBS-EP buffer at a flow rate of 10 ul/minute for 1 hour. Following stabilization of the sensorchip surface, 1 ug of recombinant *Encephalitozoon cuniculi* MsAP was manually applied to a single flow well at 5 ul/min for 20 minutes using the Kinject function. The flow well was washed with HBS-EP at a flow rate of 10 ul/minute until the sensorchip surface stabilized. Solutions of heparin, chondroitin sulfate A, dextran sulfate, and dextran were prepared in the HBS-EP buffer to a final concentration of 100 ug/ml. One hundred microliters of each carbohydrate solution was injected stepwise over the flow well surface at a flow rate of 10 ul/minute. A total of 10 micrograms of each carbohydrate was injected over the flow well surface using the Kinject function. The flow well surface was regenerated using two short pulses of Gentle Antibody/Antigen wash solution (Pierce) after injection of each carbohydrate solution. The wash solution strips bound exogenous carbohydrate from the chip surface with little, in any, effect on the immobilized EcMsAP. Data are given in resonance units and as a function of R_{max} , or the theoretical maximum amount of each carbohydrate capable of binding the immobilized EcMsAP.

Results

Identification of a Putative Microsporidia Host Cell Binding Protein

The host cell-binding assay was used to determine which, if any, microsporidia spore proteins interact with the host cell surface. Western analysis of biotin labeled *Encephalitozoon cuniculi* spore protein shows a ~40kDa band that binds the surface of *in vitro* grown host cells (Figure 4A; lane 5; arrow). The host cell-binding assay was repeated with biotin labeled *E. intestinalis* spore protein revealing a slightly larger band that also binds the host cell surface (Figure 4B; lane 5; arrow). Also included on each blot is total spore protein before and after TCA precipitation. Each blot includes a negative control showing host cell proteins that interact with alkaline phosphatase conjugated streptavidin; the host cell background reaction must be subtracted from the sample lanes containing host cell and microsporidia spore protein.

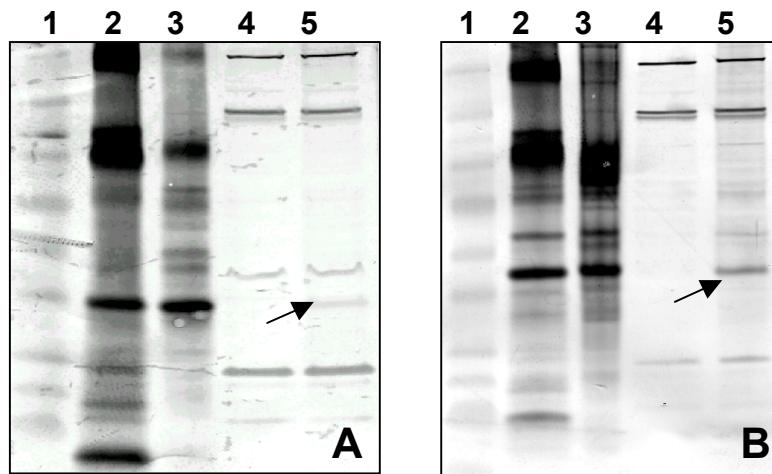


Figure 4.1 Identification of *Encephalitozoon cuniculi* and *E. intestinalis* host cell binding proteins. Proteins were incubated on the host cell surface for 2 hours followed by thorough washing. Host cell and spore proteins were resolved by SDS-PAGE followed by transfer to nitrocellulose. Proteins were labeled with alkaline phosphatase conjugated streptavidin followed by detection with NBT/BCIP. Also included on each blot is a protein size standard (lanes 1), total spore protein before (lanes 2) and after (lanes 3) TCA precipitation, host cell protein negative control (lanes 4), and host cell protein with bound spore protein (lanes 5). Arrow shows molecular weight band that was excised from a Coomassie stained gel for MALDI-TOF.

MALDI-TOF and Database Analysis

To identify the microsporidial protein of interest, *Encephalitozoon cuniculi* spore protein was resolved by SDS-PAGE followed by Coomassie staining. The Coomassie stained band corresponding to the ~40kDa *E. cuniculi* protein of interest was excised and identified using MALDI-TOF mass spectrometry.

Database analysis revealed several *E. cuniculi* proteins in the excised gel band that were screened for conserved motifs that may be involved in adhesion. The amino acid sequence of each *E. cuniculi* gene identified was evaluated for highly conserved heparin-binding motifs characterized by 'XBXBBX' or 'XBXBBXB,' where "X" is any neutral amino acid and "B" is a basic amino acid. Candidate proteins were also screened for the integrin-binding motif (-RGD-).

A single *E. cuniculi* gene (ECU01_0820) was discovered which contained two heparin-binding motifs and an integrin-binding motif (Figure 4.2).

Coincidentally, previous analysis of an *E. intestinalis* cDNA library revealed a homolog of the ECU01_0820 gene. Sequence analysis of the *E. intestinalis* gene revealed three heparin-binding motifs and an integrin binding motif at the conserved location (Figure 4.2).

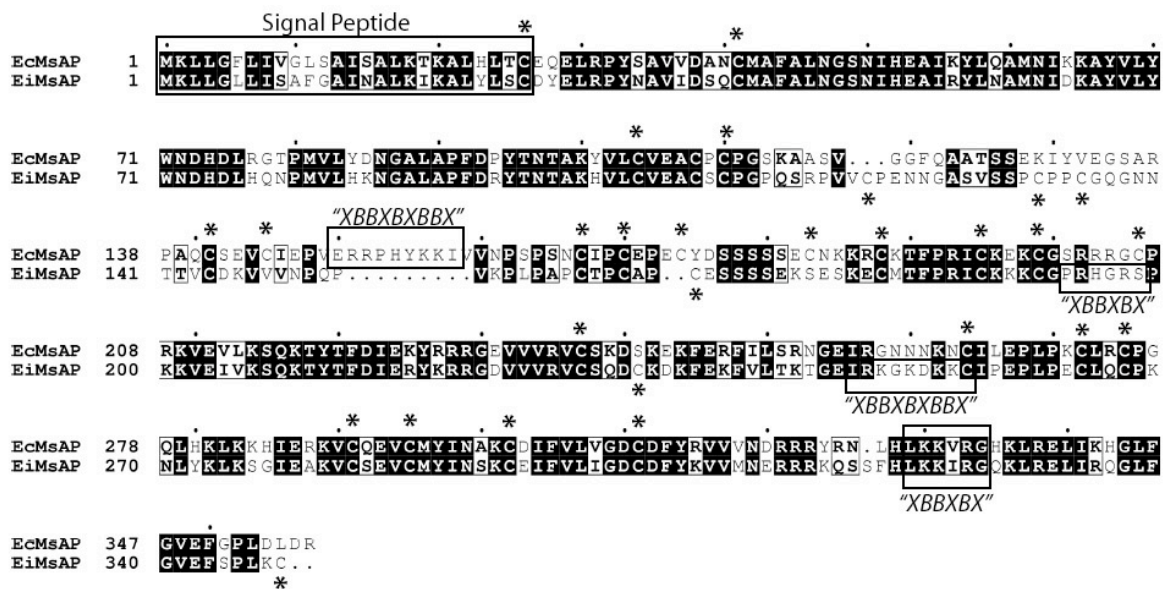


Figure 4.2 Amino acid sequence alignment of *Encephalitozoon intestinalis* microsporidia adherence protein (EiMsAP) and the *E. cuniculi* microsporidia adherence protein (EcMsAP). A consensus sequence is also shown. Conserved amino acids are shaded in black and similar amino acids are shaded in gray. Heparin-binding motifs for each amino acid sequence are outlined. The basic amino acids ('B') and neutral amino acids ('X') of each heparin-binding motif are labeled. Cysteine residues are indicated by asterisks. Every 10th amino acid is indicated by a dot.

Western Analysis with Purified EcMsAP Rabbit Antibodies

Rabbit polyclonal antisera were generated against heterologously expressed EcMsAP. Antibodies from pre-bleed and final bleed serum samples were Protein A/G purified and used in Western analysis to confirm reaction with recombinant EcMsAP as well as native EcMsAP and EiMsAP from microsporidia spore lysates (Figure 4.3). Western analysis revealed that the purified antibodies react with a ~42 kDa recombinant EcMsAP (Figure 4.3A; lane 2) as well as a ~40 kDa EcMsAP (Figure 4.3A; lane 3) and ~40 kDa EiMsAP (Figure 4.3B; lane 1) from microsporidia spore lysates.

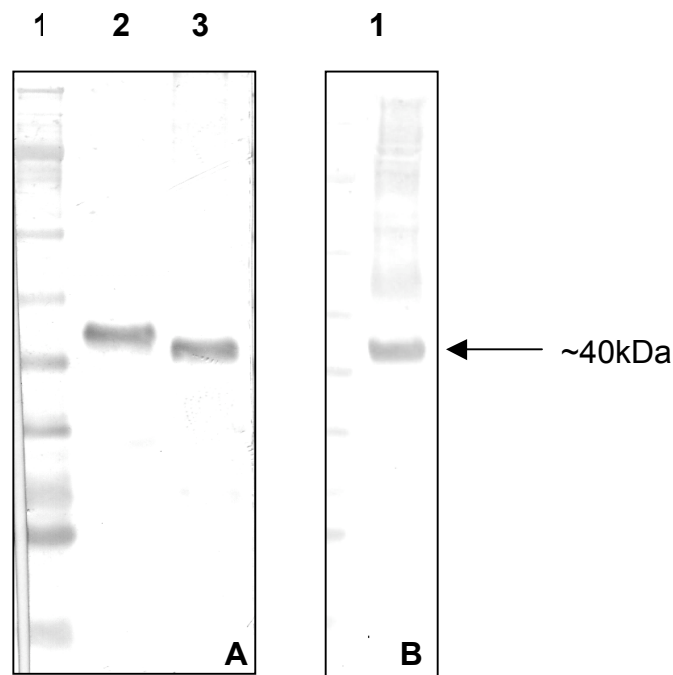


Figure 4.3 Western analysis of recombinant EcMsAP (A; lane 2), *Encephalitozoon cuniculi* spore lysate (A; lane 3), and *E. intestinalis* spore lysate (B; lane 1) using Protein A/G purified EcMsAP antibodies. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The blot was blocked overnight with 5% nonfat dry milk. The proteins were reacted with the protein A/G purified EcMsAP antibodies followed by reaction with an alkaline phosphatase conjugated goat anti-rabbit secondary antibody. Proteins were detected with NBT/BCIP.

MsAP Localization

Purified EcMsAP antibodies were used to determine localization of MsAP in host cells infected with *Encephalitozoon cuniculi* spores using immuno-gold labeling and transmission electron microscopy (Figure 4.4). Immuno-TEM analysis of immature and mature microsporidial forms shows that MsAP is present on all developmental forms of *E. cuniculi* including meronts (M), sporonts (SP), sporoblasts (SB), and mature spores (MS) (Figure 4.4A). EcMsAP is also located in the cytoplasm of meronts and sporonts, as well as on the developing plasma membrane (Figure 4.4B). Mature spore forms show high levels of immuno-gold labeling throughout the spore wall including the plasma membrane, endospore, and exospore (Figure 4.4C).

Purified EcMsAP antibodies were also used to determine localization of MsAP in host cells infected with *Encephalitozoon intestinalis* spores (Figure 4.5). Immuno-TEM analysis of *E. intestinalis* infected rabbit kidney cells shows mostly mature forms including the sporoblast and mature spore (Figure 4.5A). The most prominent immuno-gold labeling of the *E. intestinalis* sporoblast (Figure 4.5B) and mature spores (Figure 4.5C) is associated with the endospore and exospore.

Also of interest, MsAP is heavily localized to the polar sac anchoring disk region of mature *Encephalitozoon* spores (Figure 4.6). This is the first microsporidial protein identified that shows anchoring disk localization. A second microsporidial protein, MADAM, is also localized to the anchoring disk region of *E. cuniculi* and *E. intestinalis* spores (unpublished).

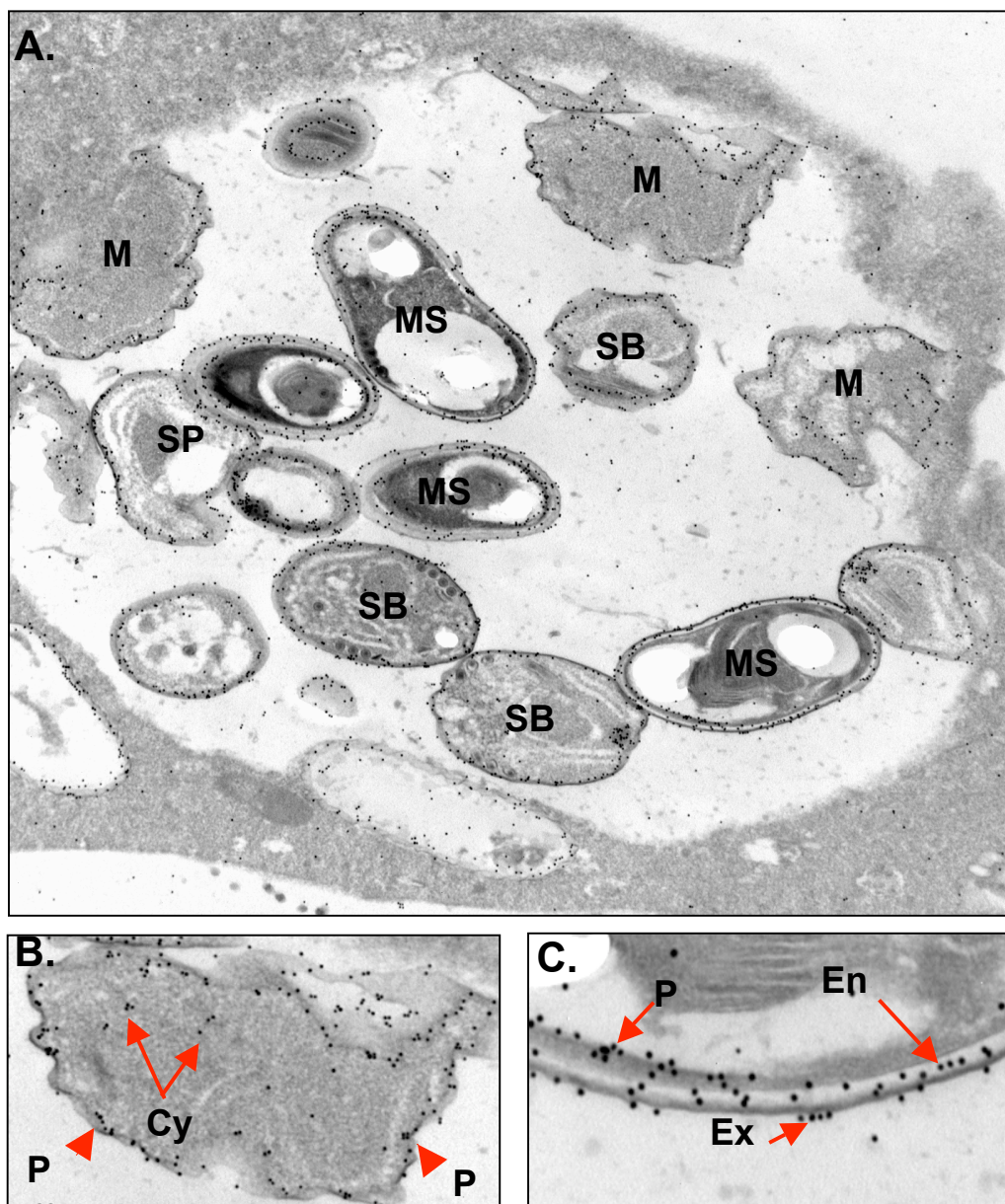


Figure 4.4 Immuno-transmission electron micrographs of developing *Encephalitozoon cuniculi* within a parasitophorous vacuole (A), a meront (B), and the spore wall of a mature spore (C). Lowicryl embedded *E. cuniculi* infected host cells were sectioned and blocked using BSA. Sections were then reacted with purified EcMsAP antibodies followed by labeling with 10 nm gold particles conjugated to anti-rabbit antibodies. Sections were viewed using a Tecnai 10 (FEI) transmission electron microscope (M = meront; SP = sporont; SB = sporoblast; MS = mature spore; Cy = cytoplasmic localization; En = endospore localization ; Ex = exospore localization; P = plasma membrane localization).

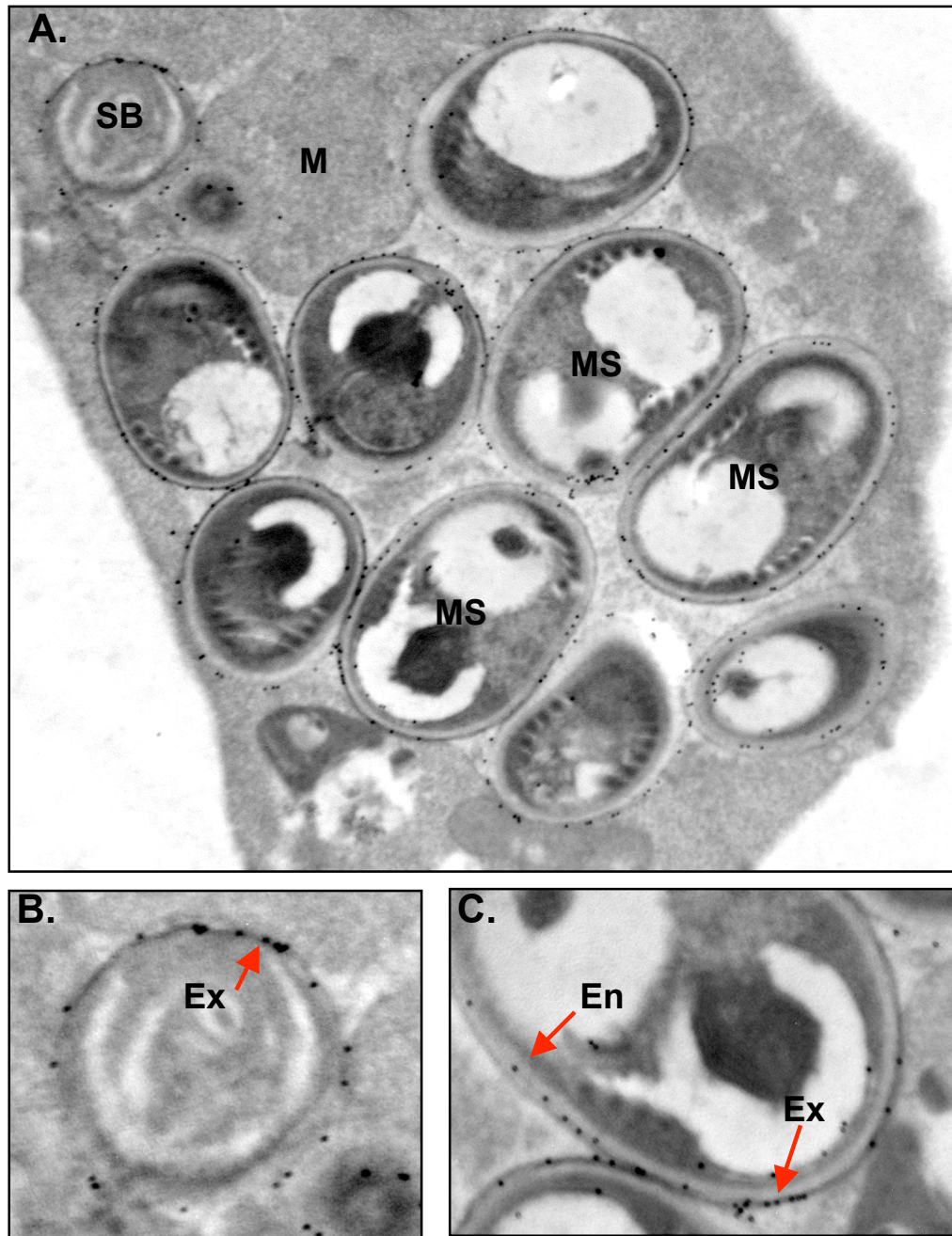


Figure 4.5 Immuno-transmission electron micrographs of developing *Encephalitozoon intestinalis* parasites within a parasitophorous vacuole (A), a sporoblast (B), and the spore wall of a mature spore (C). Lowicryl embedded *E. intestinalis* infected host cells were sectioned and blocked using BSA. Sections were then reacted with purified EcMsAP antibodies followed by labeling with 10 nm gold particles conjugated to anti-rabbit antibodies. Sections were viewed using a Tecnai 10 (FEI) transmission electron microscope (M = meront; SB = sporoblast; MS = mature spore; En = endospore localization; Ex exospore localization).

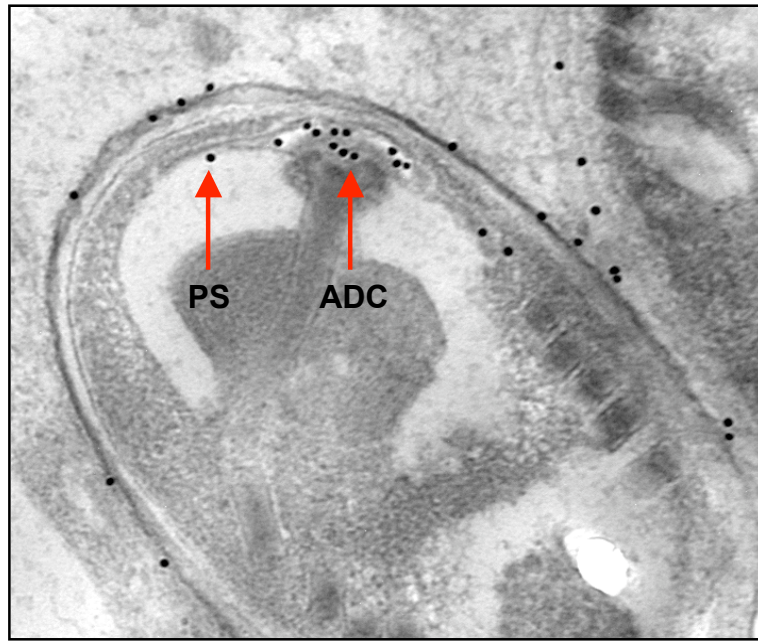


Figure 4.6 Localization of MsAP to the anchoring disk complex (ADC) and polar sac (PS) of an *E. intestinalis* mature spore. Lowicryl embedded microsporidia infected host cells were sectioned and blocked using BSA. Sections were then reacted with purified EcMsAP antibodies followed by labeling with 10 nm gold particles conjugated to anti-rabbit antibodies. Sections were viewed using a Tecnai 10 (FEI) transmission electron microscope.

Inhibition of Spore Adherence and Host Cell Infection by Recombinant EcMsAP

A series of *in vitro* assays were used to determine if recombinant EcMsAP influences spore adherence and host cell infection. Also included in these assays were recombinant EcMsAP mutant proteins created using site directed mutagenesis. Site directed mutagenesis was used to sequentially remove the two heparin-binding motifs of the parent EcMsAP gene resulting in proteins lacking heparin-binding motif #1 (HBM1DM), heparin-binding motif #2 (HBM2DM), both heparin-binding motifs (DDM), and an N-terminal deletion mutant (NTDM) used as a control (Figure 4.7).

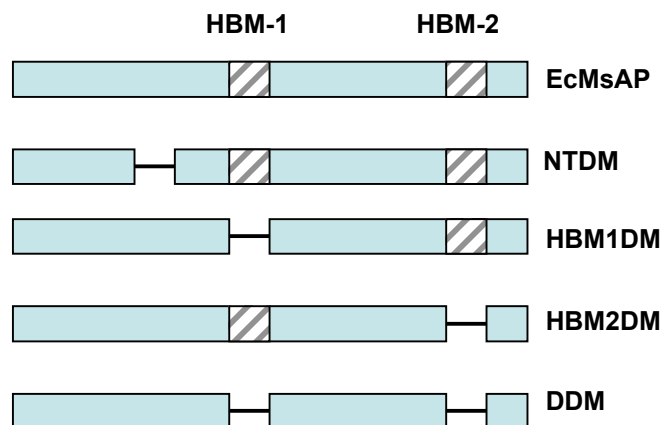


Figure 4.7 Diagram of the parent EcMsAP construct including deletion mutants created by site directed mutagenesis. Mutagenesis was used to sequential remove heparin-binding motif #1 (HBM-1) and heparin-binding motif #2 (HBM-2). The resulting constructs include the N-Terminal Deletion Mutant (NTDM), used as a control in spore adherence and infectivity assays, a Heparin-binding motif #1 Deletion Mutant (HBM1DM), a Heparin-binding motif #2 Deletion Mutant (HBM2DM), a and the Double Deletion Mutant.

Spore adherence assays with the parent EcMsAP construct and the deletion mutants indicate that recombinant EcMsAP inhibits *E. intestinalis* spore adherence to host cells by almost 70% at a concentration of 1 ug/ml (Figure 4.8A). The NTDM and HBM2DM proteins also inhibit spore adherence in a dose dependent manner, effectively mimicking recombinant EcMsAP. HBM1DM and DDM proteins did not influence spore adherence at protein concentrations up to 1 ug/ml, the highest concentration assayed. These data indicate that recombinant EcMsAP losses all ability to inhibit spore adherence following deletion of heparin-binding motif #1. Deletion of heparin-binding motif #2 had no effect on spore adherence inhibition suggesting it is not an active heparin-binding motif or that it is not an exposed epitope of the recombinant protein.

A series of *in vitro* assays was also conducted to determine the influence of recombinant EcMsAP and the EcMsAP mutants on host cell infection (Figure 4.8B). Addition of EcMsAP, NTDM, and HBS#2DM proteins during the spore adherence portion of the assay each reduced infectivity by over 60% at a concentration of 1 ug/ml. However, HBD#1DM and DDM proteins did not significantly influence infectivity compared to the NTDM control. Infectivity assay data mirror results from the spore adherence assay and support the conclusion from previous studies that spore adherence directly influences host cell infection. These data also support the hypothesis that native EcMsAP participates in spore adherence to host cell surfaces. Finally, spore adherence and infectivity assay data suggest that heparin-binding motif #1 is responsible for most if not all of the binding ability of recombinant EcMsAP.

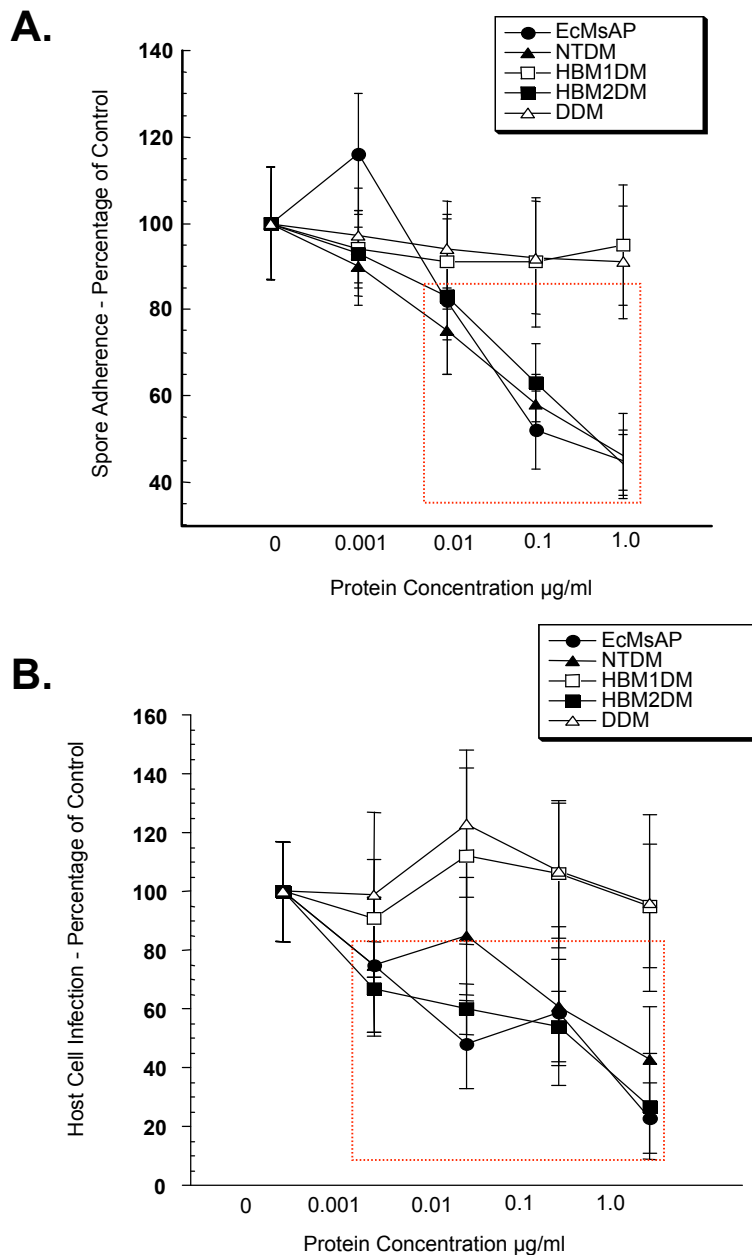


Figure 4.8 Spore adherence (A) and host cell infection (B) assays with recombinant EcMsAP and the EcMsAP deletion mutants. Each recombinant protein was incubated on individual host cell monolayers in the presence of *Encephalitozoon intestinalis* spores. Multiple dilutions of each protein were evaluated. For the spore adherence assay, monolayers were washed, fixed, and an immunofluorescence assay was performed to visualize microsporidia spores. Spores were counted using fluorescence microscopy. For the infectivity assay, monolayers were washed, incubated in supplemented DMEM for an additional 36 hours, washed, fixed, and stained with Uvitex. Host cells were evaluated for infection using UV microscopy. Data are representative of three independent experiments (n=20). The Student's t-test was used to determine significance. Data boxed in red show significant difference as compared to a control without the addition of recombinant protein ($p < 0.0001$).

Inhibition of Spore Adherence and Host Cell Infection by EcMsAP Specific Antibodies

In vitro assays were also used to assess the influence of EcMsAP antibodies on *Encephalitozoon intestinalis* spore adherence and host cell infection (Figure 4.9). Spore adherence assay data indicate that purified EcMsAP specific antibodies inhibit spore adherence to host cells in a dose dependent manner at dilutions from 1:10⁵ to 1:10³. Maximum spore adherence inhibition of 56% was attained with a 1:1000 dilution of EcMsAP antibodies. Host cell infection was also inhibited following the addition of purified EcMsAP antibodies during the spore adherence portion of the infectivity assay. Infectivity assay data indicate that antibody dilutions from 1:10⁵-1:10³ inhibit host cell infection in a dose dependent manner. Maximum infectivity inhibition of 46% was attained with a 1:1000 dilution of EcMsAP antibodies.

These data suggest the EcMsAP is spore wall associated protein that potentially interacts with the host cell during spore adherence. Inhibition of spore adherence and infection with EcMsAP specific antibodies indicates that antibodies could potentially be used as a therapeutic agent to block spore adherence to the surface of enterocytes. Finally, these data support the use of EcMsAP as a potential vaccine candidate to prevent microsporidiosis.

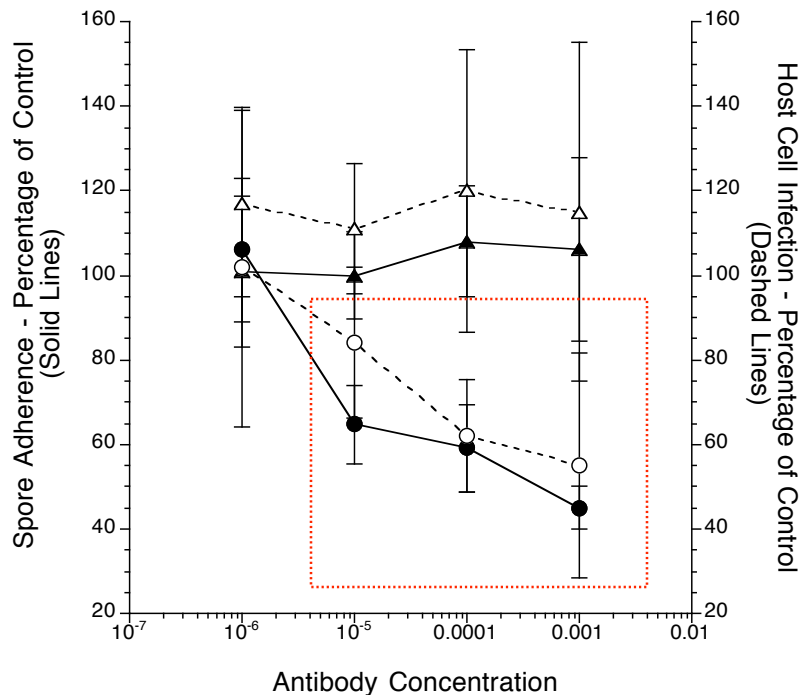


Figure 4.9 Spore adherence and host cell infection assays with purified EcMsAP antibodies. Dilutions of EcMsAP specific antibodies were incubated on individual host cell monolayers in the presence of *Encephalitozoon intestinalis* spores. Dilutions from $1:10^6$ - $1:10^3$ were evaluated. For the spore adherence assay, monolayers were washed, fixed, and an immunofluorescence assay was performed to visualize microsporidia spores. Spores were counted using fluorescence microscopy. For the infectivity assay, monolayers were washed, incubated in supplemented DMEM for an additional 36 hours, washed, fixed, and stained with Uvitex. Host cells were evaluated for infection using UV microscopy. Data are representative of three independent experiments (n=20). The Student t-test was used to determine significance. Data in the red box show significance compared to the control ($p < 0.0001$).

Recombinant EcMsAP Binds the Host Cell Surface

The host cell-binding assay was used to evaluate the ability of recombinant EcMsAP to bind to the surface of cells grown *in vitro* (Figure 4.10). Western analysis using purified EcMsAP antibodies indicates that recombinant EcMsAP binds the surface of Vero cells as well as Chinese hamster ovary cells (CHO-K1). Recombinant EcMsAP exhibits attenuated binding to the heparin deficient CHO cell line pgsA-745, as well as the GAG-deficient CHO cell line

pgsD-677. An *Encephalitozoon cuniculi* total spore protein sample was included as a positive control. CHO cell and Vero cell total protein samples were included as negative controls. Negative control lanes represent host cell monolayers that were not incubated with recombinant EcMsAP.

Host cell-binding assay data illustrate that recombinant EcMsAP binds the surface of host cells *in vitro*. Inability of recombinant EcMsAP to bind GAG-deficient cells indicates that EcMsAP most likely interacts with glycans on the host cell surface. These data support the hypothesis that spore wall associated EcMsAP is the lectin responsible for microsporidia spore adherence to host cell glycans.

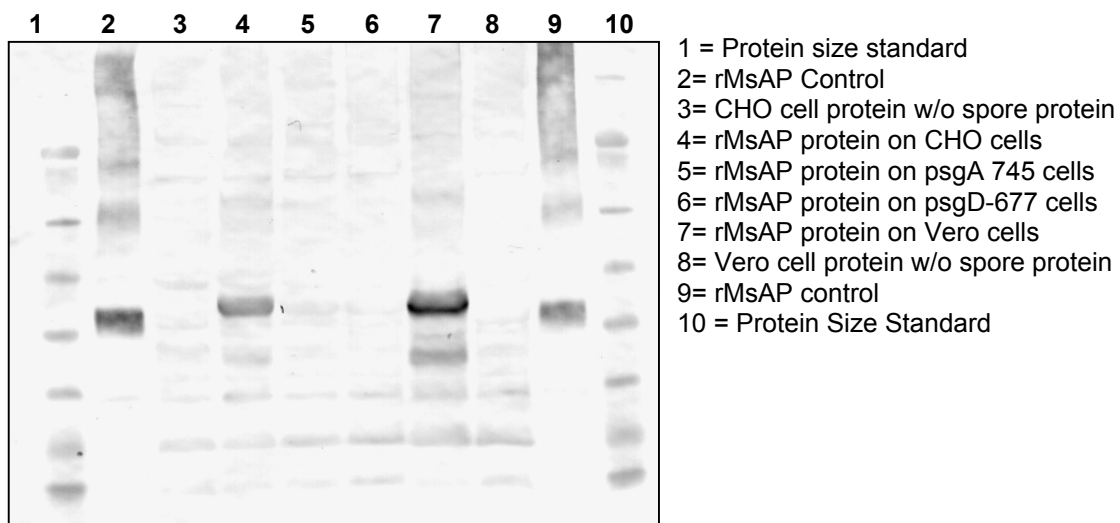


Figure 4.10 Analysis of recombinant EcMsAP attachment to the surface of host cells grown *in vitro*. One microgram of recombinant EcMsAP was applied to confluent host cell monolayers grown in 12-well plates. Monolayers were incubated for 2 hours at room temperature followed by washing with PBS. Host cell and bound spore protein was solubilized in SDS-Boiling Buffer followed by SDS-PAGE. Protein was transferred to nitrocellulose and the blot was blocked overnight in 5% non-fat dry milk in TBS. The blot was washed in TBST and was incubated in purified EcMsAP antibodies (1:500 in TBST) for 2 hours. The blot was washed in TBST and was incubated for 1 hour in an alkaline phosphatase conjugated goat anti-rabbit secondary antibody (1:2000 in TBST). The blot was washed in TBST and detected using NBT/BCIP (Pierce).

Sulfated Glycans Bind Recombinant EcMsAP

Biomolecular interactions analysis (BIA) was used to evaluate the molecular interactions between recombinant EcMsAP and exogenous glycans (Figure 4.11). These interactions were evaluated by measuring the surface plasmon resonance of each molecular interaction in real-time. Data are presented as resonance units (RUs) and as a percentage of R_{Max} , or the theoretical maximum binding capacity of immobilized EcMsAP for each glycan. RUs and R_{max} was calculated from baseline data collected before and after the injection of each glycan. Sharp peaks on the sensorgram indicate an RU shift in response to sensorchip surface regeneration using the Gentle Antibody/Antigen wash solution (Pierce). The sensorchip surface was regenerated following injection of each carbohydrate. Although complete regeneration of the chip surface was never attained between carbohydrate injections, the chip surface was stabilized providing accurate baseline readings. While not an uncommon BIA complication, incomplete regeneration of the sensorchip surface could influence the total resonance unit shift. However, incomplete sensorchip surface regeneration should not otherwise influence the molecular interaction between immobilized EcMsAP and the exogenous glycans.

BIA data indicate that 1242 resonance units of EcMsAP were immobilized on the CM5 sensorchip surface. Ultimately, 231 RUs of dextran sulfate (47% of R_{max}), 483 RUs of CSA (31% of R_{max}), 956 RUs of heparin (86% of R_{max}), and 0 RUs of dextran (0% of R_{max}) were bound to the immobilized recombinant EcMsAP. These preliminary data indicate that the sulfated GAGs heparin and

CSA interact with immobilized recombinant EcMsAP. The sulfated carbohydrate dextran sulfate also interacts with immobilized EcMsAP, but non-sulfated dextran does not. Not only does BIA confirm our hypothesis that EcMsAP is a lectin, it provides the first line of direct evidence that the recombinant protein possesses glycan binding ability.

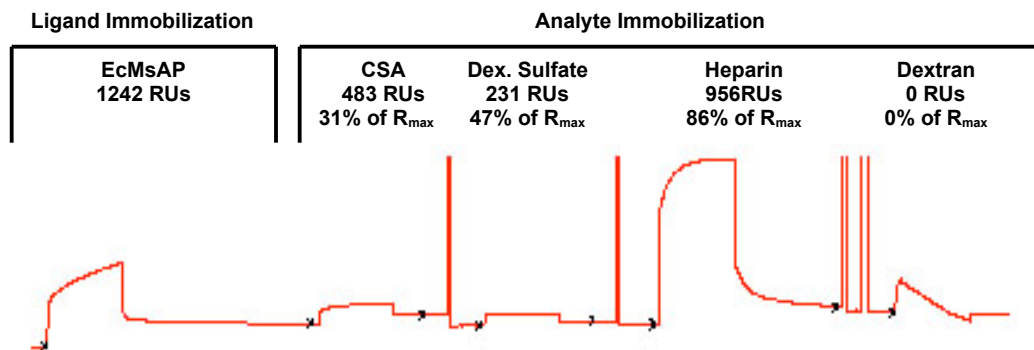


Figure 4.11 Biomolecular interactions analysis sensorgram showing recombinant EcMsAP binding by exogenous glycans. A tetra-histidine epitope tag monoclonal antibody was covalently linked to the surface of a CM5 sensorchip. Recombinant EcMsAP was immobilized by the antibody and glycans were injected over the CM5 sensorchip surface. Interaction between recombinant EcMsAP and each glycan is indicated by a positive shift in resonance units. Glycan binding data are given as R_{max} values, or the theoretical maximum amount of each glycan that can bind recombinant EcMsAP immobilized on the CM5 sensorchip surface.

Discussion

Attachment of obligate intracellular pathogens to the host cell surface is often the first step in a series of events leading to infection and propagation. Viral, bacterial, fungal, and parasitic pathogens employ this tactic, usually resulting in receptor-mediated endocytosis or active invasion. Microsporidians of the genus *Encephalitozoon*, specifically *E. cuniculi* and *E. intestinalis*, adhere to

host cell surfaces prior to and during host cell infection. However, microsporidia spores typically do not enter the host cell during infection. Instead, a hollow polar tube is discharged from the apical end of the spore that ultimately penetrates the host cell cytoplasmic membrane. The polar tube is then used to transfer infectious sporoplasm into the host cell cytoplasm, giving rise to a new generation of spores (Wittner 1999).

Recent examination of microsporidia spore adherence to *in vitro* grown host cells indicates that microsporidia spores bind glycosaminoglycans (GAGs) on the host cell surface (Hayman et al. 2005; Leitch et al. 2005). These studies also indicate that inhibition of GAG mediated adherence by exogenous GAGs reduces host cell infection. Host cell infection is highly attenuated in the GAG-deficient Chinese hamster ovary cell lines pgsA-745 and pgsD-677. Chemical inhibitors of host cell GAG synthesis and GAG sulfation also reduce spore adherence and infection (Hayman et al. 2005). A recent study also indicates that the divalent cations manganese and magnesium activate a spore surface constituent leading to a dose-dependent increase in spore adherence to host cell surfaces. Consequently, augmentation of spore adherence by manganese and magnesium contributes to a significant increase in host cell infection (Southern et al. 2006). Unfortunately, little else is known about the molecular events that govern the early stages of host cell infection, including host cell recognition. The goal of this study was to identify and characterize microsporidia spore protein(s) that interact with the host cell surface.

A host cell-binding assay was designed to determine if microsporidia

spore proteins interact with the host cell surface. This experiment yielded a single ~40kDa *Encephalitozoon cuniculi* spore protein that was eventually identified as ECU01_0820, a hypothetical gene with no known function. ECU01_0820 is known here as *Encephalitozoon cuniculi* Microsporidial Adherence Protein (EcMsAP). A highly conserved *E. intestinalis* homologue of EcMsAP was recovered from a subtractive cDNA library. The conserved nature the EcMsAP and EiMsAP genes suggests a critical function for these proteins.

Coincidentally, ECU01_0820 (EcMsAP) was recently described by Peuvrel-Fanget and colleagues as strictly an endospore protein and was consequently named Endospore Protein 1 (EnP1) (Peuvrel-Fanget et al. 2006). However, our immuno-TEM analysis shows that, in addition to the endospore, MsAP is clearly localized to the exospore region of the spore wall. This suggests that MsAP is exposed to the extra-spore environment and could potentially interact with constituents associated with the host cell surface. Furthermore, our immuno-TEM analysis indicates that every developmental form of *E. cuniculi* possesses MsAP, including immature microsporidial forms that lack a defined endospore. Meronts and sporonts show MsAP localization in the cytoplasm as well as the developing plasma membrane. Cytoplasmic localization likely indicates protein precursors associated with ribosomes, the endoplasmic reticulum, or vesicles bound for the plasma membrane. MsAP is also localized throughout the spore wall of *E. cuniculi* sporoblasts and matures spores including the plasma membrane, endospore, and exospore. MsAP is also detected in abundance on the anchoring disk and polar sac regions of mature *E. cuniculi*

parasites. Immuno-TEM analysis using *E. intestinalis* infected host cells shows similar patterns in MsAP distribution. MsAP is predominantly associated with the spore wall of *E. intestinalis* sporoblasts and spores. As with *E. cuniculi*, MsAP is also localized to the anchoring disk and polar sac regions of the mature *E. intestinalis* spore.

Progression of MsAP localization from the cytoplasm of immature microsporidia forms sequentially through the plasma membrane, endospore/exospore, and the anchoring disk of mature spores may illustrate developmental distribution of this protein. Concentration of gold labeling in the endospore region of mature spores suggests that MsAP may be a structural protein involved in supporting or maintaining the highly cross-linked chitinous endospore. With multiple heparin binding domains, exposure of MsAP on the exospore may explain the affinity of spores for sulfated glycans such as heparin and chondroitin sulfate A. Surface exposure of the MsAP heparin binding domains may allow interaction with host cell proteoglycans resulting in spore “tethering” to the host cell surface. This interaction may facilitate other binding events, possibly with host cell integrins, or may solitarily provide the necessary stimulus to initiate polar tube extrusion and host cell infection. Surface exposure of MsAP may also facilitate spore adherence to matrix-associated glycosaminoglycans potentially contributing to dissemination throughout host tissues.

Spore adherence and infectivity assay data support the hypothesis that MsAP is surface exposed and involved in spore adherence to the host cell

surface. Both the EcMsAP recombinant protein and EcMsAP specific antibodies inhibited spore adherence and reduced host cell infection. Sequential deletion of the EcMsAP heparin binding domains significantly influenced the ability of recombinant EcMsAP to inhibit spore adherence and infection. Deletion of the first *E. cuniculi* heparin-binding motif removed all inhibitory property from recombinant EcMsAP suggesting a critical function for this amino acid sequence. Deletion of the second heparin-binding motif did not influence the ability of EcMsAP to inhibit spore adherence. This suggests that the second heparin-binding motif does not participate in the adherence event or is not an exposed epitope of MsAP. Unfortunately, the uniqueness of this protein precludes any assumptions about its structure.

Host cell-binding assay data also support the hypothesis that MsAP is located on the spore wall and interacts with the host cell surface. Recombinant EcMsAP bound the surface of *in vitro* grown Vero and CHO cells. As with intact *Encephalitozoon* spores, the recombinant protein could not be washed from the surface of the host cell once bound. This suggests avid binding between the recombinant protein and the host cell receptor. Host cell-binding assays with the CHO mutant cell lines pgsA-745 and pgsD-677 indicates that MsAP interacts with host cell glycans. Recombinant EcMsAP bound to CHO cells in a manner similar to Vero cell binding but exhibited attenuated binding to the glycan-deficient CHO cell lines. These data confirm prior results showing attenuated spore adherence to glycan-deficient CHO cells lines (Hayman et al. 2005).

Finally, biomolecular interactions analysis (BIA) data indicate that recombinant EcMsAP interacts with exogenous heparin, chondroitin sulfate A, and dextran sulfate. The carbohydrate dextran did not bind to immobilized recombinant EcMsAP. These data show definitively that recombinant EcMsAP possesses glycan binding ability. To this point all information supporting spore adherence to host cell glycans has been indirect. Data gathered using BIA provides directed evidence that recombinant EcMsAP is a glycan binding protein. BIA suggests that the glycan binding ability of *Encephalitozoon* spores is, at least in part, attributed to spore wall MsAP. Furthermore, preliminary evidence suggests that the addition of EDTA during BIA reduces glycan interaction with immobilized recombinant EcMsAP. Additionally, presence of manganese during BIA increases the amount of glycan that binds the immobilized EcMsAP (data not shown). Pending further analysis, these data may confirm that EcMsAP is in some way activated by manganese. Collectively, BIA data indicate that EcMsAP may be the lectin on the spore surface that facilitates spore adherence to host cell glycans.

Although several novel microsporidia spore proteins have been described in the past few years, few have any described function. Instead of a rapid-fire protein identification approach, we selected a single novel protein and not only determined its localization but also examined its function using a series of *in vitro* assays. Unfortunately, the absence of a genetic system for the microsporidia prevents knockout or knockdown analysis of MsAP. Even without such definitive studies, this research shows localization of MsAP throughout the sporewall, and

argues the point that MsAP (EnP1) is not strictly an endospore protein. This study also shows that recombinant EcMsAP inhibits spore adherence and host cell infection. This research also shows that recombinant EcMsAP possesses the ability to bind the surface of *in vitro* grown host cells. Most importantly, this research reaffirms the importance of spore adherence to host cell infection and supports our hypothesis that spore adherence is integral to spore activation and host cell infection.

Acknowledgments: We thank Dr. Priscilla Wyrick and Ms. Judy Whittimore and the ETSU Transmission Electron Microscope Core Facility for the consultation and technical assistance for TEM. We also thank the College of Graduate Studies at East Tennessee State University for funding in support of this project.

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CHAPTER 5

CONCLUSION

Microsporidia are receiving increasingly more attention due to their classification as emerging pathogens of importance to human health. Thanks to modern electron microscopy, the intracellular developmental cycle of several pathogenic microsporidia species is well defined (Wittner 1999). Microsporidial ultrastructure throughout intracellular development is also described providing insights into these unique fungal pathogens (Wittner 1999). Unfortunately, little information is available regarding the molecular events that govern basic microsporidia biology, including spore adherence, spore activation, host cell infection, intracellular development, nutrient acquisition, subversion of host cell resources, inhibition of host cell apoptosis, and parasite egress from the dying host cell. A better understanding of these activities at the molecular level will likely provide new avenues for the development of novel therapeutics to treat or even prevent human microsporidiosis.

Our laboratory is most interested in deciphering the molecular mechanisms that mediate spore adherence. Our research strategy was designed to (1) elucidate the host cell receptor that participates in spore adherence, (2) evaluate the effects of divalent cations on spore adherence, and (3) identify and characterize ligands on the spore surface that mediate spore adherence to host cells.

The goal of our first study was to determine the specific host cell receptors that participate in spore adherence *in vitro* (Hayman et al. 2005). Numerous viruses, bacteria, parasites, and fungi bind host cell surface glycans to facilitate infection or colonization. Our initial hypothesis was that microsporidia employ a similar tactic by exploiting glycans associated with the host cell surface. A series of *in vitro*, commercially available exogenous glycosaminoglycans were used as potential inhibitors of spore adherence. The GAGs heparin, heparan sulfate, chondroitin sulfate A, and chondroitin sulfate B each inhibited spore adherence to the surface of *in vitro* grown host cells. The non-GAG carbohydrates dextran sulfate and mucin also inhibited spore adherence in a dose dependent manner. In all cases, spore adherence inhibition was dose dependent but complete inhibition was never achieved. The non-sulfated carbohydrates dextran and hyaluronic acid did not inhibit spore adherence suggesting that glycan sulfation and structure are important in the spore adherence process. Collectively, these assays suggested that host cell GAGs may be exploited by microsporidia during spore adherence.

Several other approaches were taken to evaluate the role of GAGs during microsporidia spore adherence. Chinese hamster ovary (CHO) cell lines deficient in surface-expressed GAGs were used in a series of spore adherence assays along with a non-mutant parent CHO cell line. These data show a significant decrease in spore adherence to the mutant CHO cells that lack surface expressed GAGs. A chemical inhibitor of GAG expression and a chemical inhibitor of GAG sulfation were also used to examine the role of host

cell GAGs in spore adherence. The GAG inhibitor p-nitrophenyl- β -D-xylopyranoside inhibited spore adherence when host cells were incubated in the compound prior to the spore adherence assay. The GAG sulfation inhibitor sodium chlorate also inhibited spore adherence when host cells were incubated in the compound prior to the spore adherence assay. Finally, by modifying the spore adherence assay, we were able to show that host cell infection is directly influenced by adherence of microsporidia spores to the host cell surface. Infectivity assay data revealed that a decrease in spore adherence contributes to reduced infection. This report was not only the first to describe spore adherence in terms of a molecular mechanism, it also illustrated a direct relationship between spore adherence and host cell infection.

The goal of our second study was to evaluate the influence of divalent cations on spore adherence and host cell infection (Southern et al. 2006). The divalent cations manganese, magnesium, and calcium are important cofactors known to influence adherence of pathogenic organisms to the host cell surface. Divalent cations also play an important physiological role in cell-to-cell and cell-to-matrix interactions, especially among integrins. Our initial hypothesis was that divalent cations would augment spore adherence and infection *in vitro*. Our initial studies support our hypothesis by showing a dose dependent increase in spore adherence attributed to manganese and magnesium. Calcium, however, did not influence spore adherence to the host cell surface. When used in the infectivity assay, all three divalent cations contributed to an increase in host cell infection. This is not surprising for manganese and magnesium because both divalent

cations also contributed to an increase in spore adherence. Interestingly, calcium did not contribute to increased spore adherence but did cause and increase in host cell infection. Data from infectivity assays using calcium support a decades-old study that illustrates the importance of calcium ion flux to polaroplast swelling and polar tube discharge.

A series of spore adherence and infectivity assays were also conducted with divalent cations and the chelator EDTA. When included in the *in vitro* assays, EDTA abrogated all divalent cation induced spore adherence and host cell infection. These data suggest that the observed increase in spore adherence and infection is attributed to the presence of divalent cations in the assay buffer and is not an artifact of this assay system. Finally, a series of spore adherence assays was performed to determine whether a host cell or spore constituent was being activated by manganese. Numerous permutations of the spore adherence assay were conducted with host cells and spores that were (1) pretreated with manganese or not pretreated, (2) fixed with paraformaldehyde or not fixed, and (3) treated with manganese at the time of the spore adherence assay or not treated. This series of spore adherence assays indicated that the divalent cation manganese activates a constituent on the spore surface and not the host cell surface. Data from this study not only confirmed a direct relationship between spore adherence and host cell infection, it illustrated that spore adherence is a dynamic event that is activated by divalent cations.

The goal of our third and most recent study was to determine which, if any, microsporidia spore wall associated proteins participate in spore adherence

to host cells *in vitro*. To answer this fundamental question, we adapted a commonly used assay to evaluate the adherence of individual microsporidia spore proteins to the surface of a host cell monolayer. This assay revealed a single protein from *Encephalitozoon cuniculi* spore lysate that binds the host cell surface. This protein could not be washed from the surface of the host cell monolayer suggesting avid binding with a host cell receptor. The microsporidia host cell binding protein was visualized using Western analysis and was ultimately identified from a Coomassie stained gel band using mass spectrometry. The gene was identified as ECU01_0820, known hereafter as *Encephalitozoon cuniculi* Microsporidia Adherence Protein (EcMsAP). Knowing the microsporidia spores bind host cell GAGs, the EcMsAP gene was screened for heparin-binding motifs as well as the integrin-binding motif. Analysis of the EcMsAP amino acid sequence revealed two heparin-binding motifs and a single integrin-binding motif.

The EcMsAP gene was eventually cloned and recombinant protein was heterologously expressed. The histidine-tagged fusion protein was used to raise antibodies in rabbits that were previously identified as naive to microsporidia infection. Protein A/G purified antibodies from pre-bleed and terminal bleed antisera samples were used to evaluate localization of native MsAP in host cells infected with *E. cuniculi* or *E. intestinalis*. Immuno-transmission electron microscopy revealed that MsAP is associated with every developmental form of *E. cuniculi* and is associated with the cytoplasm, plasma membrane, endospore,

exospore, and anchoring disk. MsAP is also localized to the same structures in *E. intestinalis* sporoblasts and mature spores.

Recombinant EcMsAP was used in a series of *in vitro* assays to determine the influence of the protein on spore adherence and host cell infection. Data from these assays indicate that recombinant EcMsAP is a potent inhibitor of spore adherence and infection. Site-directed mutagenesis was then used to determine amino acids critical to the function of EcMsAP as a spore adherence inhibitor. Site-directed mutagenesis was used to sequentially remove the two heparin-binding sites resulting in gene constructs that lacked heparin-binding motif #1, heparin-binding motif #2, and a construct that lacked both heparin-binding motifs. An N-terminal deletion was also engineered to determine if deletion mutagenesis unduly influenced protein function. All four mutant constructs were used in spore adherence and infectivity assays along with EcMsAP parent construct. In short, spore adherence and infectivity assay data revealed that deletion of the first heparin-binding motif abolished all ability of EcMsAP to inhibit spore adherence and host cell infection. The N-terminal deletion mutant and the heparin-binding motif #2 deletion mutant inhibited infection similar to the parent EcMsAP construct. Similarly, protein A/G purified EcMsAP antibodies were used in the spore adherence and infectivity assays. Data from these assays indicate that EcMsAP specific antibodies are also potent inhibitors of spore adherence and host cell infection. Protein A/G purified antibodies generated against a microsporidial heat shock protein did not inhibit spore adherence, providing a needed negative control (data not shown).

Recombinant EcMsAP was also used in the host cell-binding assay to determine if the heterologously expressed protein could bind to the surface of *in vitro* grown host cells. Western analysis revealed that the recombinant EcMsAP binds Vero and CHO cells but does not bind the glycan-deficient CHO cell lines pgsA-745 and pgsD-677. Host cell-binding assay data confirm previous findings that show reduced spore adherence to the glycan deficient cell lines.

Finally, recombinant EcMsAP was used in the highly quantitative biomolecular interactions analysis, also known as surface plasmon resonance (SPR). SPR data indicate that heparin, chondroitin sulfate A, and dextran sulfate bind EcMsAP with a clear preference for heparin; non-sulfated dextran did not bind EcMsAP. These data are the first to directly show glycan interaction with recombinant EcMsAP and suggest that native *E. cuniculi* and *E. intestinalis* MsAP may also bind glycans, including the glycans associated with the host cell surface. Furthermore, these data agree with several previously published studies showing heparin, CSA, and dextran sulfate, but not dextran, as potent inhibitors of spore adherence.

The three studies described here provide unique insights into the molecular events that govern microsporidia spore adherence to host cells surfaces. These studies also provide evidence supporting a direct relationship between adherence and host cell infection. Furthermore, our studies indicate that the physical interaction between spores and the host cell surface may be the stimulus required to activate microsporidia spores ultimately leading to polar tube discharge and host cell infection.

A better understanding of the molecular biology of microsporidia spore adherence and infection is an irreplaceable resource in a quest to develop novel therapeutics to treat or even prevent microsporidiosis. Thankfully, the basic molecular biology of the microsporidia is now receiving attention from research groups worldwide. A continued focus on the molecular biology of microsporidia will almost assuredly provide better insights into this “curious” group of organisms.

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B.A. Biology, University of North Carolina at Asheville,
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Encephalitozoon intestinalis to host cells *in vitro*. *Infect Immun* 73(2): 841-8.
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adherence and host cell infection by divalent
cations. *FEMS Microbiol Lett* 260(2): 143-9.
- Honors and Awards: Graduate Student Research Grant
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